PAGE
363
367
377
379
387
396
404
410
416

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THE CHANGE FROM BENIGN TO MALIGNANT IN CHEMICALLY INDUCED WARTS IN MICE.

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MANY warts, resulting from repeated painting of mice with tar, had been found (Mottram, 1934, 1935, 1936*a*) to be of mixed constitution, so that when many small pieces from the same wart were autografted, tumours resulted varying from simple epithelial cysts to solid malignant growths. Serial sections of such warts often showed local difference in morphology, differentiation, abundance of mitoses, sharpness of the basal layer and invasion of the dermis, thus confirming a mixed constitution. It seemed therefore that the gradual change from benign to malignant, often described, represented the gradual overgrowth of the benign parts by the more rapidly growing malignant. This explanation was supported by finding that the growth rate of such warts gradually increased, as would be the case when warts were gradually coming to consist more and more of rapidly growing cells at the expense of slowly growing (Mottram, 1936*b*).

This gradual change from benign to malignant has been cited as evidence of a step-up process for the genesis of malignancy; but the above observations do not support this. On the other hand, some recent observations, here described, give an alternative explanation.

An abundant crop of warts can be produced in mice by single paintings of benzpyrene, if supplemented with pre- and post-paintings of croton oil (Mottram, 1944*a*, *b*). These warts differ in several respects from those following repeated paintings; a much smaller percentage is malignant; the malignant warts are malignant from the start, and the change from benign to malignant is not seen, no matter how long post-painting with croton oil be continued.

EXPERIMENTAL.

I. Six mice were painted on both flanks on days 0, 2, 4, 6 and 8 with 1 per cent. croton oil in acetone and on day 9 with 0·03 c.c. of 0·3 per cent. benzpyrene in acetone; subsequently both flanks were painted thrice weekly with croton oil, and in addition the left flank once a week with benzpyrene. The results are shown in Fig. 1. It is seen that for 15 weeks the tumour production with the single painting of benzpyrene was equal to that of the repeated paintings, but that later it fell behind. The single

painting method gave rise to only one malignant tumour, the repeated paintings to five malignant tumours in 18 weeks, all starting as benign warts. The spreading widely of these malignant warts made counts beyond 18 weeks valueless.

These observations at once suggest that the change from benign to malignant, seen in repeated paintings, is due to the production of malignant cells by action of benzpyrene on the benign cells during the repeated paintings. Thus is visualized, not a step-up process, but the occurrence within a benign wart of a change similar to that which sometimes causes malignant tumours to arise upon the epithelium without first passing through the stage of a benign wart.

This explanation is easily tested by taking a set of benign warts produced by the single painting method, which will not become malignant, and repeatedly painting them with benzpyrene to see if malignancy supervenes.

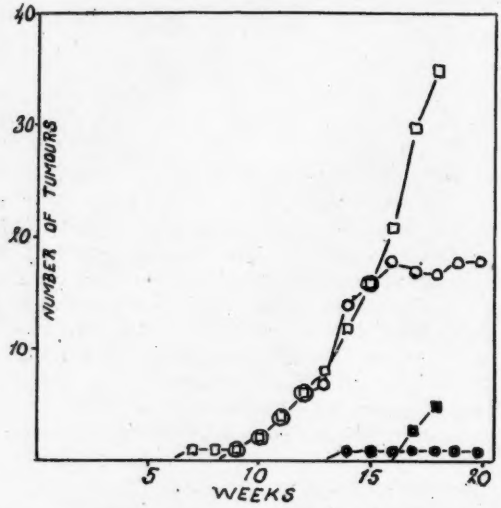

FIG. 1 shows the weekly occurrence of tumours resulting from single paintings of benzpyrene (circles) and repeated paintings (squares). White circles and squares = benign warts, and black = malignant. For further details see text.

II. Mice were painted on days 0, 2, 4, 6 and 8 with 1 per cent. croton oil in acetone on both flanks ; on day 9 the flanks were treated with 0·03 c.c. of 0·3 per cent. benzpyrene in acetone and subsequently with croton oil thrice weekly. The resulting warts were measured weekly, and when they had grown to such a size that their benign nature was patent they were painted once a week with benzpyrene using a fine brush, care being taken as far as possible to paint only the wart ; the croton oil painting was continued thrice weekly. When the warts were thought to have become malignant or when the animal died the warts were histologically examined, cutting serial sections and mounting one section in twenty.

Altogether 22 warts were thus treated. Eleven of these remained benign after painting with benzpyrene for from 10 to 30 weeks. Five became malignant, showing extensive invasion of underlying muscle. Six were considered to be locally malignant and a short description of these follows :

(1) A pedunculated wart : Grew slowly until it reached 24 sq. mm. ; had then been painted with benzpyrene for 11 weeks ; became ulcerated, and in 3 weeks diminished

to 9 sq. mm. Subsequently regrew, extended deeply and bled. Killed 7 weeks later. Histology: Solid fingers of growth extending deeply into the dermis, but with well defined basal layer; at centre small ulcerated area, and beneath this all regular epithelial structure lost; instead polymorphic spindle-shaped cells are penetrating deeply into the dermis. Diagnosis: Local malignancy. At one place on the edge of the wart a third kind of growth is present, of small dark basal cells forming small cell masses with no keratinization. Diagnosis: Resembling a rodent ulcer.

(2) A horny wart of 19 sq. mm.: After 7 weeks' painting measured 324 sq. mm. It was ulcerated but did not extend deeply. Killed. Histology: A horny growth with well-defined basal layer. At two places this was replaced by spindle cells fanning out into the dermis. Diagnosis: Probably locally malignant.

(3) A pedunculated wart: It became sessile at 17 weeks. Killed at 20 weeks. Histology: Much surface keratin; thick epithelium with well-defined basal layer. In central region oat-shaped cells descending deeply into the dermis, almost to the muscles. Diagnosis: Local malignancy.

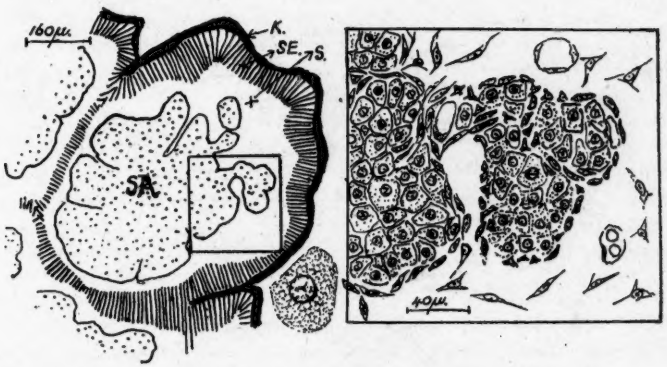

FIG. 2.—On the left is shown a portion of the much-folded benign wart. Its stroma (s) is invaded by a sebaceous adenoma (sa). k = keratin; se = squamous epithelium of the benign wart. On the right is an enlarged drawing of a part of the adenoma lying in the sparsely cellular stroma of the wart. The stroma of the adenoma is scanty and very cellular; its glandular cells show sebaceous characters, a large circular nucleus and an abundant foamy cytoplasm, seen in the cell separately drawn.

(4) A sessile wart: No growth for 8 weeks; then rapid growth for 8 weeks; deep extension at 14 weeks. Histology: A horn with very cellular base and well-defined basal layer. At one edge a growth of small undifferentiated cells fading off into a rich cellular stroma; at one place muscle is invaded. Diagnosis: Locally malignant.

(5) A sessile wart: 10 weeks old when first painted; no signs of malignancy; 5 weeks later was ulcerated and bleeding. Killed 9 weeks. Histology: Keratinizing wart with well-defined basal layer. There is a central region where small spindle cells are spreading deeply into the dermis. Diagnosis: Locally malignant.

(6) A sessile wart: Grew from 10 to 52 sq. mm. in 23 weeks; deep extension at 14 weeks and haemorrhage at 15 weeks. Histology: A much folded squamous wart with a simple epithelium. In the stroma of this wart is growing a sebaceous adenoma which occupies nearly the whole wart (see Fig. 2). At one place the squamous epithelium is greatly thickened; the cells are large and very polymorphic, and they spread deeply into the dermis without any defined basal layer. Diagnosis: Locally malignant.

The results show that out of 22 warts treated, 5 became malignant, 6 showed local signs of malignancy, and 11 remained entirely benign. Such changes were not found in benign warts resulting from single paintings of benzpyrene, even when examined long after their first appearance.

DISCUSSION.

The change of warts from benign to malignant had previously been shown (literature cited) to be due to their multiple constitution, but how this constitution came about remained unexplained. It is now seen to be due to the repeated paintings causing the formation of malignant cells among those of the benign wart.

Recent experiments (Mottram, 1944*a*, *b*) showed that the skin of mice can be sensitized to benzypyrene by pre-painting with croton oil which causes active proliferation of the epidermis, and de-sensitized by pre-treatment with cantharidin which inhibits proliferation. In benign warts, especially those growing rapidly, proliferation is proceeding more rapidly than in conditions of hyperplasia, and therefore benign warts would be expected to be very sensitive to the production of new tumours in them by further paintings of benzpyrene. New benign warts in existing benign warts are likely to remain undetected; on the contrary, new malignant tumours will soon manifest themselves. Thus is visualized, not a step-up process, but repeated hits by the benzpyrene on a sensitive tissue, any one of which is capable of producing, and may result in a malignant tumour.

There are many papers describing the effect of painting, with cyclic hydrocarbons, benign tumours produced by viruses and in other ways. It is not proposed to load this paper with a detailed consideration of these experiments, of which the most recent is by Rous and Friedewald (1944). Briefly, the conclusion is that the benign tumours become rapidly cancerous, and often in a multiple way. It seems that these very rapidly growing benign tumours are extremely sensitive; showing once again (see Mottram, 1944*b*) that benzpyrene acts especially on proliferating cells.

In naturally occurring tumours in man, the change from benign to malignant is often seen and sometimes one type of malignant tumour turning into another—for instance a rodent ulcer becoming a squamous epithelioma. Whether this is due to the continued presence of a tumour-producing agent is unknown. The experimental results described indicate that benign tumours in man should be treated in order to prevent cancer.

CONCLUSIONS.

An explanation is given for the frequent change from benign to malignant of warts produced by painting mice with tumour-producing substances. This change is due to the repeated paintings, often initiating new localized malignant changes within them. Thus their multiple constitution, previously observed, is accounted for, and the gradual change from benign to malignant shown to represent the gradual replacement of the slow-growing benign by the fast-growing malignant cells. The theory that this change is due to a step-up process in the genesis of cancers is thus not supported.

REFERENCES.

MOTTRAM, J. C.—(1934) *Amer. J. Cancer*, **22**, 801.—(1935) *J. Path. Bact.*, **40**, 407.—(1936*a*) *Ibid.*, **42**, 79.—(1936*b*) *Amer. J. Cancer*, **28**, 155.—(1944*a*) *J. Path Bact.*, **56**, 181. (1944*b*) *Ibid.*, **56**, 391.

ROUS, P., AND FRIEDEWALD, W. F.—(1944) *J. exp. Med.*, **79**, 511.

INHIBITION OF THE CARCINOGENIC ACTION PRODUCED BY A WEAKLY CARCINOGENIC HYDROCARBON ON A HIGHLY ACTIVE CARCINOGENIC HYDROCARBON.

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EXPERIMENTS aimed at the inhibition of carcinogenesis can be divided into two groups: (1) Those which try to stop the progress of a cancer already established; these are very numerous, since they include therapeutic attempts of all kinds. The most recent work in this direction includes the experiments of Haddow (1935) using carcinogenic compounds to diminish tumour growth, and those of Boyland and Boyland (1940) using a great number of amines, sulphamides and various nuclear poisons.

(2) Those attempting to delay or to prevent tumour formation; it is these that we have to consider here and of which there are only a few examples. Berenblum (1929, 1931) has shown that the addition of 0·1 per cent. of mustard gas (dichlorodiethylsulphide) to the tar used to paint mice, prevents papilloma formation, although the epidermis undergoes the usual hyperplasia and the growth of tumours already established is not checked. Reimann and Hall (1936) using sulphydrylated compounds (like parathiocresol) alternately with 1 : 2 : 5 : 6-dibenzanthracene on the skin of mice have observed a diminution in the percentage of tumours produced by this hydrocarbon.

We report here some instances of an inhibitory effect noted in the course of some experiments in carcinogenesis. Starting with the idea that the mutation which transforms a normal cell into a malignant cell is the result of the alteration of an organic substrate by the fixation to it of some toxic molecules (in this case polycyclic hydrocarbons), we proposed to use with this compound another hydrocarbon of little or no toxicity, but with a molecular structure resembling the first as closely as possible, and the fixation of which in the cell should protect it to some extent against the cancerization.

EXPERIMENTAL.

The two first experiments already published (Lacassagne, Buü-Hoi and Cagniant, 1944; Lacassagne, Buü-Hoi, Daudel and Rudali, 1944) will be recalled and then the results of others will be given.

The mice used all belonged to two selected strains of *L'Institut du Radium* (Strains XVII and R III),* and were young adults (3–5 months) of both sexes weighing from

* Skin epitheliomata generally appear earlier in the R III strain with similar conditions of treatment, but owing to the poor resistance of this strain, a high and early mortality leads to trouble and we have used the XVII strain when possible.

12–20 g. In spite of the difficulties caused by the occupation the food of the animals (black bread and oats) was sufficient in amount. In every experiment the agents were applied to the interscapular region of two batches of 10 mice ; in one batch a solution of the carcinogenic compound alone ; in the other the same solution in which was dissolved an equal quantity of a weakly carcinogenic hydrocarbon. The animals were observed until they died spontaneously, or when possible they were killed when moribund to obtain good histological material.

Experiment I.—Dibenzofluorene and Methylcholanthrene.

A. *Benzene solution.*

Twenty mice of the XVII strain were used. One drop of a benzene solution containing 0·5 per cent. of methylcholanthrene was applied to the interscapular region of the 10 control mice and 0·5 per cent. of methylcholanthrene + 0·5 per cent. of 1 : 2 : 5 : 6-dibenzofluorene (equimolecular proportions) to the other 10 mice. The treatment was stopped after 63 applications in 21 weeks.

(*a*) In the controls which were treated with the pure methylcholanthrene solution, epilation occurred between the 7th and 12th day in the treated area and soon extended further. The first papilloma appeared on the 46th day (average the 79th day), and distinct signs of malignancy from the 79th day (average 108th) ; the general condition

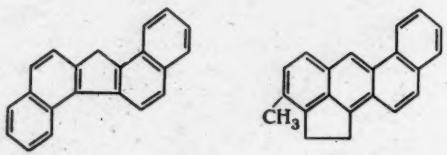


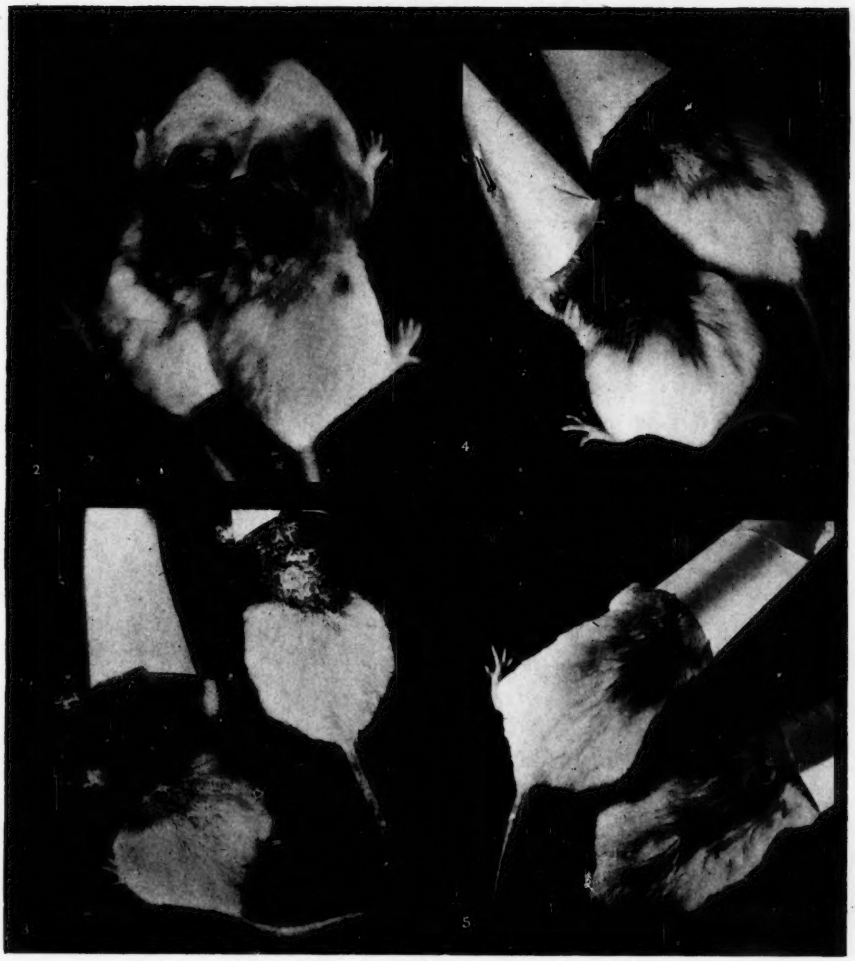
FIG. 1.—(a) 1 : 2 : 5 : 6-dibenzofluorene. (b) 20-methylcholanthrene.

of the mice rapidly deteriorated with cessation of growth and wasting (average weight before autopsy 16·95 g.). All 10 animals died bearing cutaneous epitheliomas, two of them even before the end of the applications (120 and 127th day) ; the longest survival was 171 days (average survival 152 days).

The characteristic visceral lesions consisted chiefly in hypertrophy of the spleen and of the liver, with at first reticular hyperplasia, then leucocytic infiltration of more or less myeloid type, and lastly para-amyloid degeneration as described by Beltrami (1935). These changes were already present on the 124th day, and increased in gravity in proportion to the length of survival. Lung adenoma was first observed on the 152nd day.

(*b*) The pathological changes were more irregular, and appeared later in the animals treated with the double solution. Of the first 6 that died, one of bronchopneumonia at the 82nd day and the others of intoxication between the 151st and the 182nd day, only one (dead on the 152nd day) has shown histological evidence of epithelioma ; two others had papillomata. The last 4 mice dying between 200 and 274 days had all developed malignant tumours, although the ulcerated skin lesion in the last mouse was so small that it was not observed, and cancer was only revealed by epitheliomatous invasion of a lymph gland. The general state of the animals remained satisfactory for a longer time (average weight before the autopsy 17·5 g. Average survival 191 days).

Similarly the visceral lesions varied considerably from one animal to another, but



FIGS. 2–5.—State of the skin of the most affected and the least affected of the treated animals. Figs. 2, 3: Methylcholanthrene alone. Figs. 3, 4: Methylcholanthrene + dibenzofluorene. Figs. 2, 4: 103 days after the beginning of the treatment. Figs. 3, 5: 163 days after the beginning of the treatment.

were usually less severe than in those observed in the control batch, in spite of the longer survival. Lung adenoma was first observed at the 200th day.

B. *Acetone solution.*

The same experiment was repeated, but this time with acetone solutions, containing the respective quantities of only 0·3 per cent. of the two hydrocarbons and with 10 mice of the R III strain, receiving two applications in a week (this technique was adopted in most of the subsequent experiments). The results obtained are summarized in Table I.

TABLE I.

Pure methylcholanthrene.

	Number of days.				
(*a*) Appearance of papillomata . . .	—	—	31	53	92
(*b*) Appearance of carcinoma	—	—	130	79	94
(*c*) Death	10	50	148	152	154

Methylcholanthrene + dibenzofluorene.

	Number of days.				
(*a*) Appearance of papillomata . . .	—	—	79	106	113
(*b*) Appearance of carcinoma	—	—	100	109	138
(*c*) Death	59	70	167	168	201

Compared with Experiment Ia, the earlier appearance of cancers and the earlier death is certainly connected with the different reaction of the two strains of mice. The phenomenon of inhibition, however, although less pronounced (perhaps because of the lower concentration of the solutions), is found again since the average times of appearance of papillomata, cancer and of death were respectively 59, 101 and 103 days for the controls and 99, 119 and 153 days for the animals treated with the mixture of the two hydrocarbons.

Experiment II.—1 : 2 : 5 : 6-Dibenzacridine and 1 : 2 : 5 : 6-Dibenzanthracene.

To 20 female mice of the R III strain weighing about 20 g. three paintings weekly with a brush impregnated with a fatty vehicle containing 0·3 per cent. of 1 : 2 : 5 : 6-dibenzanthracene were given for the control batch and 0·3 per cent. of 1 : 2 : 5 : 6-dibenzacridine + 0·3 per cent. of dibenzanthracene for the 10 other mice. The applications were continued until the appearance of a well-established cutaneous cancer.

In the two batches the cutaneous reactions were, of course, less severe, and appeared later than in the previous experiment on account of the less irritant excipient, as well

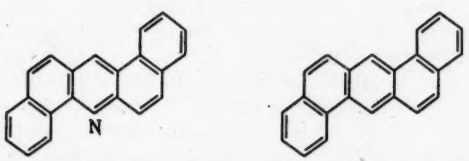


FIG. 6.—(a) 1 : 2 : 5 : 6-dibenzacridine. (b) 1 : 2 : 5 : 6-dibenzanthracene.

as the lower concentration and lower toxicity of the carcinogenic hydrocarbon. However, the difference between the animals of the first and the second batch was equally clear.

(*a*) In the controls, treated with the pure dibenzanthracene solution, epilation began on the treated region after nearly one month; it was complete 15 days later, and remained defined except in three animals where only a temporary growth of scattered hair was observed after 2½ to 3 months. Crusts usually preceded the appearance of the papillomata. The graph (Fig. 7) shows that the average weight of the animals had diminished two months after the beginning of the treatment. The dates of the appearance of papillomata and of carcinomata are also indicated. Cancerization occurred in all cases between the 105th and the 198th day. No animal survived beyond the 263rd day; death was caused as much by wasting as by progress of the cancer.

At autopsy considerable enlargement of the spleen (average of 372 mg. for 3 animals dead about the 212th day) and of the liver (1260 mg.) was noted. However,

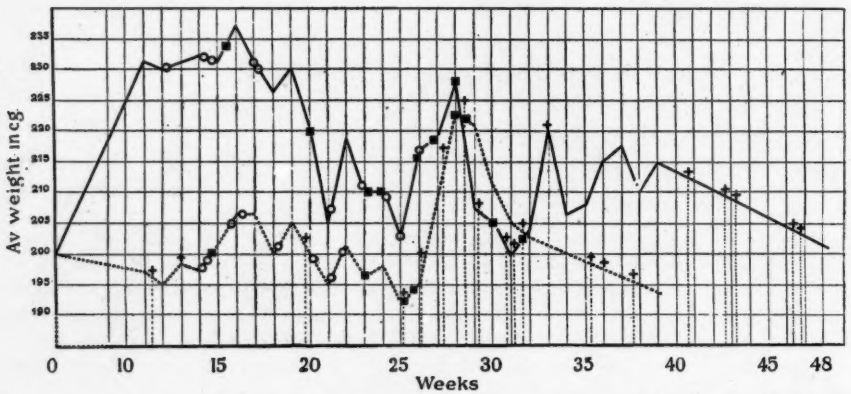


FIG. 7: ——— = average weight of the surviving animals after treatment with 1 : 2 : 5 : 6-dibenzacridine + 1 : 2 : 5 : 6-dibenzanthracene. ..... = average weight of the surviving animals after treatment with 1 : 2 : 5 : 6-dibenzanthracene alone. O = Appearance of the first papilloma. ■ = Malignant transformation. + = Death.

even localized para-amyloid degeneration was met with only in the two last animals dead on the 257th and 263rd day. No lung adenoma was found histologically.

(*b*) In the second batch treated with the mixture of the two hydrocarbons, the growth of the animals was not interrupted by the treatment (Fig. 7); the general condition began to deteriorate only about the 4th month after the first application. Epilation was in almost every case only temporary, a new growth of the hair appearing in the 3rd month. However, the majority of tumours began to appear at about the same time as in the controls. There was no appreciable delay in the production of papillomata and of carcinomata except in a small number of animals; the average survival, however, was appreciably longer.

In spite of the more prolonged treatment undergone by most of the mice of this batch the visceral lesions were less advanced. In the 3 animals autopsied about the 212th day, the average weight of the spleen was 217 mg. and that of the liver 1157 mg., and were thus much less than in the controls at the same time. In none of these organs, even after a treatment longer by nearly a third, have we met para-amyloid

degeneration. On the other hand, lung adenoma has been found from the 299th day, while the last animal showed a glandular generalization of its cutaneous epithelioma with lung metastasis.

Experiment III.—Chrysene and Methylcholanthrene.

Mice of the XVII strain received twice weekly a drop of a methyl-ethyl-cetone solution of 0·3 per cent. methylcholanthrene for the control batch and 0·3 per cent. methylcholanthrene + 0·3 per cent. natural chrysene for the ten other mice. The treatment was stopped after 30 applications in 15 weeks.

Table II shows the time (in days after the beginning of the treatment) of the appearance of papillomata, of carcinomata and of death for the two batches of animals.

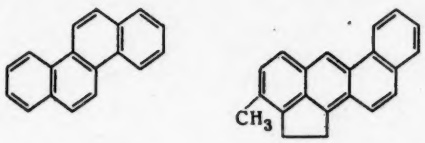


FIG. 8.—(a) Chrysene. (b) 20-methylcholanthrene.

TABLE II.

Pure methylcholanthrene.

	Number of days.									
(*a*) Appearance of papillomata . .	—	—	—	98	42	94	39	61	138	109
(*b*) Appearance of carcinoma . .	—	—	—	106	95	99	90	69	168	116
(*c*) Death	48	100	105	134	128	167	181	193	195	199

Methylcholanthrene + chrysene.

	Number of days.									
(*a*) Appearance of papillomata . .	58	125	118	99	76	90	77	90	118	99
(*b*) Appearance of carcinoma . .	126	—	120	134	132	147	122	159	133	153
(*c*) Death	126	136	169	183	203	207	211	215	223	231

This table shows weak but definite inhibition in the controls, the average times being 83 days for the appearance of papillomata, 106 for cancerization and 144 to death; in the animals treated with the mixture the corresponding periods were 95, 136 and 190 days. The average weights of the spleens and livers of six mice of each batch dying between the 126th and 207th day were found to be 270 and 1296 mg. respectively for the controls and 225 and 1050 for the others.

Experiment IV.—Methyl Derivatives of Benzacridine and Methylcholanthrene.

Six acetone solutions were prepared containing besides 0·3 per cent. of methylcholanthrene, 0·3 per cent. of one of the following compounds: 1-10-dimethyl–7 : 8-benzacridine, 2 : 10-dimethyl–7 : 8-benzacridine, 3 : 10-dimethyl–7 : 8-benzacridine, 1 : 10-dimethyl–5 : 6-benzacridine, 2 : 10-dimethyl–5 : 6-benzacridine, 1 : 4 : 10-trimethyl–7 : 8-benzacridine. They were all tested on 10 mice of the XVII strain at the rate of one drop twice weekly. The treatment was stopped after 30 applications in 15 weeks.

In every batch the general and local reactions, the papillomata and the cutaneous epitheliomata appeared in the same way, and in a manner strictly comparable with that resulting from a similar solution of methylcholanthrene alone. No inhibition was shown.

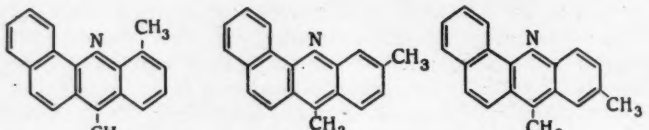


FIG. 9.—(a) 1 : 10-dimethyl–7 : 8-benzacridine. (b) 2 : 10-dimethyl–7 : 8-benzacridine. (c) 3 : 10-dimethyl–7 : 8-benzacridine. (d) 1 : 10-dimethyl–5 : 6-benzacridine. (e) 2 : 10-dimethyl–5 : 6-benzacridine. (f) 1 : 4 : 10-trimethyl–7 : 8-benzacridine.

Experiment V.—Saturation by the Dibenzofluorene Alone, Before the Application of the Mixture Dibenzofluorene and Methylcholanthrene.

Accepting the initial hypothesis (which is supported by some of the foregoing experiments) we were led to suppose that the previous application of a non-carcinogenic hydrocarbon would more clearly demonstrate its inhibitory action. Although the experiment is not yet finished, the results seem conclusive enough to be reported.

Ten mice of the XVII strain of an average weight of 16·5 g. received (from March 28, 1944) twice weekly applications of a drop of a solution containing 0·3 per cent. of dibenzofluorene in acetone. During this treatment, which lasted 40 days (12 applications), two animals died of infection. On May 9 the surviving 8 animals were in good health (23 g.), and showed in the treated areas only a limited epilation, which had been present for only 10 days. These survivors were then treated with a mixture of 0·3 per cent. methylcholanthrene and 0·3 per cent. dibenzofluorene. Four months later only 3 animals bore papillomata, which appeared between the 80th and 90th day, and of which one showed obvious malignancy on the 91st day. The skin of the 5 others showed no proliferation of the epidermis.

DISCUSSION.

The various experiments reported here provide evidence that certain weakly toxic hydrocarbons acting in competition with a highly toxic hydrocarbon on the tissues of the mouse reduce the effect of the latter and in particular delay cancerization. This is especially the case with dibenzofluorene or chrysene in association with methylcholanthrene, and also 1 : 2 : 5 : 6-dibenzacridine with 1 : 2 : 5 : 6-dibenzanthracene. This phenomenon of inhibition is observed only with some hydrocarbons, because nothing comparable has been obtained with various derivatives of benzacridine when tested with methylcholanthrene. When it occurs, the inhibition affects not only the well known local phenomena relevant to the action of a carcinogenic compound applied on the skin, atrophy of the hair-follicles, proliferation of the epidermis and finally

cancerization, but also the general and distant phenomena caused by the diffusion of the toxic agent in the organism (reactions of the reticulo-endothelial system and of the hematopoietic tissue, alterations of the hepatic and renal cells, adenomatous proliferation of the lung epithelium, etc.).

It does not seem that this inhibition can be explained by a chemical reaction between the two associated hydrocarbons, resulting in more or less complete inactivation of the carcinogenicity. Indeed, in all the instances studied here the inhibitory substance has no chemical affinity with the associated carcinogenic compound ; and it is possible to recover each constituent in an unchanged state from all the mixtures used.

There is no question either of any antagonistic action on certain enzyme systems, as can be postulated to explain the inhibition of cancer by the dichlorodiethylsulphide (diminution of glycolysis in relation to cell-respiration). Indeed, the hydrocarbons which act as inhibitors in these experiments have themselves a carcinogenic action, and act from this point of view in the same direction as the more toxic compound with which they are associated.

Another possibility is that there is a chemical reaction between the antagonist and some sulphur-containing constituent of the tissues (cysteine), as Crabtree (1940) suggests in the case of tumour growth. Experiments so far have shown that dibenzofluorene, dibenzacridine, chrysene or any of the other substances tested have at least *in vitro* no affinity for cysteine (Buü-Hoi, 1943).

It is therefore probable that this phenomenon is of a physiological nature, and that the mechanism of this inhibition must be sought in an intracellular process of a physico-chemical nature. It can be supposed that certain constituents of the substrate determining mitosis (and in the existence of which certain considerations, impossible to quote here, lead us to believe) should be capable of absorbing or at least fixing in their neighbourhood, by their secondary bonding capacity, any polycyclic molecule having certain definite morphological characteristics, of which we have just a glimpse, and the exact determination of which is one of the important tasks in the study of cancer. If, according to the theory supported by Schmidt (1941), the aromatic substance absorbed in this way presents zones with a high density of π electrons (corresponding to the presence of multiple chemical bonds) situated in accessible parts of its molecule, it could become able to catalyse the primary reaction or reactions of cancerization favouring certain quantum leaps. Quantum leaps could be produced, for example, in the complicated linkages of the protein molecule, forming the genes which determine the phenomena of heredity. If, on the contrary, the absorbed polycyclic compound does not present—or presents only to a small degree —the electronic characteristics necessary for the appearance of the catalytic property, the complex formed will have little or no effect ; the probability of activation and consequently of cancerization will be very weak.

Indeed it is easy to understand that the close analogy of configuration between the molecules of the two different hydrocarbons, intimately mixed in the same solution, will have the result that the two compounds have the same fate in the organism into which they are introduced and are retained in the same systems and organs. It is more than probable that the proportion of molecules of each substance penetrating any cell is conditioned only by the laws of probability with a coefficient of sensitivity depending on the degree of similarity of the two molecules. Thus the molecules of the inhibitory substance are absorbed by the cellular constituents along with the molecules of the strongly carcinogenic hydrocarbon, and in consequence there is a diminution in the number of complexes of high catalytic activity. The diminished absorption of the strongly carcinogenic hydrocarbon can delay the manifestation of the consequent cellular alterations. Such would be the case if the process of canceriza-

tion can be considered as a mutation due to a lesion of the substrate controlling cell division (Lacassagne, 1944).

If this is assumed it is evident that the inhibitory effect for any pair of substances will be proportional to the difference of activity of the two compounds. This is what has in fact been observed; for example, 1 : 2 : 5 : 6-dibenzacridine is less inhibitory to 1 : 2 : 5 : 6-dibenzanthracene than 1 : 2 : 5 : 6-dibenzofluorene or chrysene against methylcholanthrene, and the difference between the carcinogenic properties of the two hydrocarbons is less marked in the first pair of substances than in the second one.

These considerations, based upon experimental results, have led us to investigate non-carcinogenic substances of a structure as near as possible to those of the most carcinogenic compounds known (methylcholanthrene, 9 : 10-dimethyl–1 : 2-benzanthracene, etc.). Furthermore, starting from the idea, which seems more and more accepted, that many tumours called spontaneous are the consequence of the action (probably by a mechanism analogous to that indicated here) of certain endogenous substances, this investigation has now been extended to compounds related to sexual hormones and other steroids.

CONCLUSIONS.

Repeated applications were made on the skin of mice with solutions containing a mixture of two hydrocarbons of similar molecular configuration, but one of which is weakly and the other strongly carcinogenic, dibenzofluorene and methylcholanthrene, 1 : 2 : 5 : 6-dibenzacridine and 1 : 2 : 5 : 6-dibenzanthracene, chrysene and methylcholanthrene. In these conditions the carcinogenic power of the more active of these compounds is distinctly reduced.

The mechanism of the phenomena of inhibition of cancerization is discussed. It is supposed that the two substances, having the same affinity because of their analogy of structure, penetrate into the same cells, where they are fixed in the same substrate. Each weakly toxic molecule, by fixing itself, would hinder by its presence the fixation of a molecule of the more toxic substance, and thus delay the appearance of certain specific lesions of the cell, amongst which figures malignant transformation.

REFERENCES.

Beltrami, W.—(1935) *Tumori*, **21**, 537.
Berenblum, I.—(1929) *J. Path. Bact.*, **32**, 425.—(1931) *Ibid.*, **34**, 731.
Boyland, E., and Boyland, M. E.—(1940) *Biochem. J.*, **34**, 280.
Buü-Hoi.—(1943) *C. R. Soc. Biol.*, **137**, 725.
Crabtree, J.—(1940) *J. Path. Bact.*, **51**, 303.
Haddow, A.—(1935) *Nature*, **136**, 868.
Lacassagne, A.—(1944) *Rev. Scientifique*, **83**.
Idem, Buü-Hoi, and Cagniant, P.—(1944) *C. R. Soc. Biol.*, **138**, 16.
Idem, Buü-Hoi, Daudel, R., and Rudali, G.—(1944) *Ibid.*, **138**.
Reimann, S. P., and Hall, E. M.—(1936) *Arch. Path.*, **22**, 55.
Schmidt, O.—(1941) *Naturwiss.*, **29**, 146.

THE SIGNIFICANCE OF THE BASOPHIL CHANGES IN THE PITUITARY ACCOMPANYING VARIOUS FORMS OF THYROXINE DEFICIENCY.

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ONE of us (Griesbach, 1941) has described marked changes in the basophil cells of the anterior pituitary in rats bearing hyperplastic thyroids. Similar changes have since been observed (unpublished) to accompany thyroid hyperplasia in the sheep, rabbit, and the dog, this latter observation confirming the report of Romeis (1924) on dog goitre. From cytological evidence it was concluded that these basophil cells (" goitre cells ") were activated and not degenerate, and it seemed likely that they were in fact the site of thyrotropin production.

Griesbach and Purves (1943*a*) examined the variations of cytology and thyrotropin content produced in the pituitary under conditions of varying but quantitatively unknown thyroxine deficiency. Severe thyroxine deficiency (total thyroidectomy + lacto-vegetarian diet) was found to cause almost complete degranulation of acidophil cells as well as an increase of basophils. Moreover, severe thyroxine deficiency might well lead, secondarily, to widespread disturbances in the function of endocrines other than the thyroid, and might completely derange the capacity of the pituitary to work in a normal way (Severinghaus, 1942).

From these investigations it was therefore not possible to reach definite conclusions concerning the site of thyrotropin production. The circumstances just mentioned made it seem desirable to examine the cytological and functional responses of the pituitary to known degrees of thyroxine deficiency, which could be varied by design, in accurately measured steps.

METHODS.

The rats used were 8–10 weeks old white rats of both sexes, from a Wistar strain. They were fed throughout on a strict vegetarian diet. The mean temperature of the animal room was 65° F. Adequate amounts of iodide were added to the water. When thiourea was given it was supplied in 0·25 per cent. solution as drinking water. dl-Thyroxine was purified by recrystallization of the sodium salt from sodium carbonate solution and precipitated from hot 80 per cent. alcohol (with acetic acid). A solution was made containing dl-thyroxine 1·0 mg., sod. carb. 20 mg., sod. chloride 0·9 g., water to 100 ml. Each ml. therefore contained 10 μg. of thyroxine. The strength of the solution was checked by iodine analysis. The injections of thyroxine were started on the day of thyroidectomy and continued for 21 days, when the animals were killed. The rats were weighed daily, and the thyroxine dosage adjusted to maintain constant dosage per 100 g. of body weight. The thiourea + thyroxine treatment was carried on for 21 days. The histological activity of the thyroid tissue was measured by taking the mean acinar cell heights, as has been previously described (Griesbach and Purves, 1943*b*). The method for examination of the pituitaries has also been described in a former paper (Griesbach and Purves, 1943*a*).

Total thyroidectomy.

The trachea is exposed under ether anaesthesia. With fine scissors the isthmus is divided in the middle. The thyroid lobes are dissected out by grasping, in fine forceps, the slightly bleeding cut edge of the isthmus, freeing the lower pole and main body of the lobe by slight traction. The upper firmly attached pole, which is attacked last, can be freed by strong traction combined with blunt dissection. It is important not to grasp the tissues near the larynx for the purpose of countertraction, as this damages the recurrent laryngeal nerve. No sharp dissection should be employed in freeing the upper pole, as this will lead to a large proportion of incomplete operations. If the operation is carried out as described, it is exceptional to find any evidence of incomplete removal of the thyroid.

Subtotal thyroidectomy.

This is performed in the same way, except that two cuts are made through the isthmus at its junctions with the lateral lobes. After the removal of the latter the central part of the isthmus, scarcely visible to the naked eye, remains *in situ* on the trachea. At autopsy the ventral part of the second and third rings of the trachea, with the isthmus attached, is removed, and after fixation cut in serial sections.

RESULTS.

The results of a typical series of experiments are reproduced in Table I.

(a) *Total thyroidectomy.*

When no thyroxine was supplied, the acidophils showed the well known picture of almost complete degranulation (Severinghaus, 1937). Such pituitaries, as reported by us earlier (1943*a*), have a gelatinous appearance, evidently due to the lack of strongly refracting acidophil granules. When thyroxine—even in small dosage—was given, the acidophils were maintained in normal numbers and appearance. On the other hand, with doses of thyroxine of 2·0 μg./100 g. per day or less the basophils were increased in number, 17–37 per cent., as compared with the normal of about 5 per cent., and showed the cytological evidence of activation typical of the "thyroidectomy" or "goitre" changes. With the dose of 2·25 μg. the basophil picture changes abruptly, concerning the appearance of the cells even more distinctly than the numbers, which might still be somewhat over normal (up to 11 per cent.). The basophils with such doses (and up to 3·0 μg.) are reduced in size, the granules stain purple, there are no hyaline enclosures, the negative images of the Golgi apparatus are small,

TABLE I.—*Response of Pituitary and Thyroid Cells to Thyroxine Injections in Thyroxine Deficiency States in Rats. Average Results for Both Sexes.*

Dosage of thyroxine. μg./100 g. per day.	(*a*) Total thyroidectomy. Pituitary cells per cent.		(*b*) Total thyroidectomy + thiourea. Pituitary cells per cent.		(*c*) Sub-total thyroidectomy. Pituitary cells per cent.			(*d*) Thiourea. Pituitary cells per cent.		
	Acido.	Baso.	Acido.	Baso.	Acido.	Baso.	Thyroid cell height. μ.	Acido.	Baso.	Thyroid cell height. μ.
0	2	38	1	45	46	29	13·0	44	36	13·7
0·5	43	30	34	33	55	25	13·1	51	27	12·8
1·0	41	37	39	25	56	23	10·5	52	27	11·5
1·25	41	28	49	20	51	23	10·1	58	25	10·1
1·5	39	20	48	23	51	23	11·3	50	18	10·8
1·75	48	17	50	24	42	20	10·3	51	16	9·9
2·0	46	24	46	22	51	25	7·4	59	21	8·1
2·25	47	11	52	10	51	10	5·6	49	11	6·6
2·5	49	10	44	8	54	5	5·3	54	11	5·1
2·75	45	4	47	9	—	—	—	—	—	—
3·0	47	2	49	7	—	—	—	—	—	—

and most of the nuclei are pyknotic. The differences between the doses 2·0 and 2·25 are usually so well defined that they can be recognized at a glance through the low power lens.

(b) *Total thyroidectomy plus thiourea.*

The pituitaries of the series of animals receiving thiourea in addition to total thyroidectomy showed the transformation of the basophils from "goitre cells" to the resting type at exactly the same level of thyroxine.

(c) *Subtotal thyroidectomy.*

In these experiments the remaining thyroid tissue of the isthmus showed typical compensatory hyperplasia when no thyroxine was given. With increasing doses of thyroxine the activity of the thyroid rest, as indicated by the height of the epithelium cells—and the colloid contents—gradually decreased. At the level of 2·25 μg. thyroxine/100 g. per day the acini showed normal appearance. With the same dose the pituitary basophils were inactive, while with 2·0 μg. there were 25 per cent. basophils showing all signs of activity. The acidophils showed no significant changes throughout this series, and were present in normal percentage when no thyroxine had been injected.

(d) *Thiourea.*

After 21 days of thiourea + thyroxine treatment the results were the same as in the subtotally thyroidectomized animals. In parallel investigations the regression of the thyroid cell height to normal and the inactivation and reduction of the proportion of basophils in the pituitary was obtained with the same dosage of dl-thyroxine, viz. 2·25 μg./100 g. body weight. The acidophils appeared to be unaffected throughout the entire series.

All these experiments (groups *a–d*) have been repeated a number of times and the same general results were constantly obtained. In these immature rats there was no difference in the results obtained with the two sexes.

DISCUSSION.

The experiments of Evans, Simpson and Penchartz (1939) and of Rowlands (1942) indicated that the normal thyroxine requirement of a rat is equivalent to about 2·5 μg. dl-thyroxine per 100 g. body weight per day. This figure agrees with the amount required to inhibit thyroid hyperplasia of rats on rape seed diet (Purves, 1943), but is at variance with the amount (5·2μg. of l-thyroxine) found necessary by Dempsey and Astwood (1943) to inhibit thyroid hyperplasia in rats receiving thiouracil. Dempsey and Astwood's estimation of thyroxine requirement is based on the hypothesis that thiouracil and similar substances act by inhibiting thyroxine synthesis while leaving the thyroxine requirement completely unchanged. Recent studies with radioactive iodine (Franklin, Lerner and Chaikoff, 1944) have proved the correctness of the hypothesis that these substances depress thyroxine synthesis, but there is not sufficient evidence to prove that the thyroxine requirement is completely unchanged.

The demonstration that the calorigenic action of thyroxine is not impaired by thiourea derivatives is not sufficient evidence in this respect because the "thyroxine requirement" as estimated by Dempsey and Astwood is the amount of thyroxine needed to suppress the thyrotropin release from the pituitary.

It is necessary therefore to establish this hypothesis by determining the "thyroxine requirement" as accurately as possible by a method not involving the use of goitrogenic substances. The "thyroxine requirement" in this case means an amount of thyroxine equivalent in its effects on pituitary function to the normal daily thyroid secretion in an animal receiving adequate amounts of iodine.

The results of the subtotally thyroidectomized group allow us to make such an estimation of the normal daily requirement of thyroid secretion in terms of dl-thyroxine. These results show that the greater part of the thyroid can be taken away, and its secretion replaced by a daily injection of 2·25 μg. of dl-thyroxine per 100 g. body weight. If less is given hypertrophy occurs in the remaining thyroid fragment, indicating a thyroxine deficiency and a consequent increased release of pituitary thyrotropin. If more is given thyroid atrophy ensues, indicating thyroxine excess and a consequent inhibition of thyrotropin release. A daily supply of 2·25 μg. of dl-thyroxine per 100 g. body weight is evidently equivalent to the normal daily secretion of thyroid hormone by the thyroid of a rat under our experimental conditions.

In the subtotally thyroidectomized animals the acidophil cells of the pituitary do not show any significant change with variation of thyroxine dosage. The basophils, however, show the increase in number, size, and activity characteristic for thyroidectomized animals (Zeckwer, Davison, Keller and Livingood, 1935). These changes are present in all rats receiving 2·0 μg. of dl-thyroxine or less per 100 g. per day. This means that so long as a thyroxine deficiency is present the stimulation of the basophil cells continues. The appearance of the basophils is therefore a sensitive indicator of small degrees of thyroxine deficiency.

The results obtained after total thyroidectomy differ in only one respect from those after subtotal thyroidectomy. When no thyroxine is administered to a totally thyroidectomized animal the pituitary acidophil cells are almost completely degranulated. This response to complete thyroxine deficiency is abolished by small doses of thyroxine. The significance of this acidophil change is discussed in another paper. The basophils on the other hand continue to show the full thyroxine deficiency changes until a dose of 2·25 μg. of dl-thyroxine is reached. This confirms the result obtained from the subtotally thyroidectomized animals, and shows that the minute fragment of thyroid which was left in the latter did not secrete quantitatively significant amounts of thyroxine.

The results of the totally thyroidectomized animals treated with thiourea are the same as those obtained after thyroidectomy alone. This shows that the thiourea has no direct action on the pituitary cells. Furthermore, extrathyroidal thyroxine synthesis (Morton, Chaikoff, Reinhardt and Anderson, 1943) must be quantitatively insignificant or unaffected by thiourea administration. The sensitivity of the basophil cells is not at all altered by the thiourea administration.

We are now in a position to examine the results obtained in the animals treated with thiourea alone. The basophil changes in pituitaries of these animals can only be due to inhibition of thyroid secretion by the action of the thiourea on the thyroid. The fact that in these animals 2·25 μg. of dl-thyroxine per 100 g. per day is needed to inhibit the basophil changes shows that the inhibition of thyroid secretion by thiourea is almost complete. Thiourea administration alone never causes extensive degranulation of pituitary acidophil cells, and in this respect the action of thiourea resembles the effect of subtotal thyroidectomy rather than that of the total operation. As we shall show in another paper, this indicates that a minute amount of thyroxine continues to be secreted by the thiourea poisoned thyroid, but this amount is an insignificant fraction of the normal requirement. In addition to showing that thyroxine synthesis is almost completely suppressed, we have shown that the "thyroxine requirement" is unchanged by thiourea, and the method of Dempsey and Astwood is a valid estimation of the "thyroxine requirement," and therefore of the rate of thyroid secretion in the normal animal. We are therefore unable to explain why the result obtained by Dempsey and Astwood, viz. 5·2 μg. l-thyroxine per 100 g. per day, is so much at variance not only with our own results, but also with estimates based on

measurements of growth and metabolism (Evans, Simpson and Penchartz, 1939; Rowlands, 1942).

In investigations concerning the site of thyrotropin production it was found that in gross thyroxine deficiency an excess of thyrotropin could be detected in the blood serum (Griesbach and Purves, 1943*a*). An attempt to correlate thyrotropin production with the pituitary cytology failed because in the severe thyroxine deficiencies studied there were two cellular changes in the pituitary. In the present investigation in two groups of animals, viz. the subtotally thyroidectomized and the intact thiourea treated, thyroid tissue is present in animals with controlled thyroxine deficiencies. Moreover, we have shown that thyroid tissue under the influence of goitrogenic agents still gives a normal response curve to injected thyrotropin (Griesbach and Purves, 1943*a*). The cell height of the thyroid acinar cells can therefore be used as an indication of the level of circulating thyrotropin, and correlated with the pituitary cytology over a range of graduated thyroxine deficiency. A slight drop in thyroxine level from the normal causes a simultaneous increase in thyroid activity and increase in numbers and activity of pituitary basophils. There is no other cytological change observed in the pituitary under these conditions of slight thyroxine deficiency. We therefore conclude that the basophil cells observed in thyroxine deficiency states (" goitre cells," " thyroidectomy cells ") are the source of thyrotropin secretion. This hypothesis fits with the idea of a homeostatic mechanism for the pituitary-thyroid team, by which a small reduction in the level of circulating thyroxine evokes a vigorous specific response from the thyroid controlling cells in the pituitary long before the thyroxine level has fallen so far as to impair the function of the tissues generally.

SUMMARY.

1. The normal rate of thyroid secretion in the rat is found to be equivalent to 2·25 μg. of dl-thyroxine per 100 g. body weight per day, independent of sex.

2. The " thyroxine requirement," defined as the minimal amount of thyroxine which inhibits the thyrotropic secretory activity of the pituitary, is unaltered by the administration of thiourea. The hypothesis that the goitrogenic action of thiourea is due exclusively to almost complete inhibition of thyroxine synthesis is confirmed.

3. A slight reduction in the normal thyroxine level causes an increase in the numbers and cytological activity of the basophil cells of the pituitary. Under these conditions there is no change in the numbers or amount of granulation of the acidophil cells.

4. The activation of pituitary basophil cells in thyroxine deficiency states is correlated with increased secretion of thyrotropin. It is concluded that these basophil cells are the source of thyrotropin secretion.

REFERENCES.

Dempsey, E. W., and Astwood, E. B.—(1943) *Endocrinology*, **32**, 509.
Evans, H. M., Simpson, M. E., and Penchartz, R. I.—(1939) *Ibid.*, **25**, 175.
Franklin, A. L., Lerner, S. R., and Chaikoff, I. L.—(1944) *Ibid.*, **34**, 265.
Griesbach, W. E.—(1941) *Brit. J. exp. Path.*, **22**, 245.
Idem and Purves, H. D.—(1943*a*) *Ibid.*, **24**, 174.—(1943*b*) *Ibid.*, **24**, 185.
Morton, M. E., Chaikoff, I. L., Reinhardt, W. O., and Anderson, E.—(1943) *J. biol. Chem.*, **147**, 757.
Purves, H. D.—(1933) *Brit. J. exp. Path.*, **24**, 171.
Romeis, B.—(1924) *Virchows Arch. path. Anat.*, **251**, 237.
Rowlands, I. W.—(1942) *J. Endocrinol.*, **3**, 203.
Severinghaus, A. E.—(1937) *Physiol. Rev.*, **17**, 556.—(1942) 'Cold Spring Harbor Symposia on Quantitative Biology.' New York: Long Island Biological Association, **10**, 104.
Zeckwer, I. T., Davison, L. W., Keller, T. B., and Livingood, C. S.—(1935) *Amer. J. med. Sci.*, **190**, 145.

STUDIES ON EXPERIMENTAL GOITRE. VI. THYROID ADENOMATA IN RATS ON BRASSICA SEED DIET.

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THE negative correlation between iodine intake and the incidence of endemic goitre indicates that a low iodine intake renders the human thyroid susceptible to pathological derangement. In experimental animals iodine-deficient diets are found to produce changes in the thyroid known as thyroid hyperplasia, and characterized by loss of colloid, increased vascularity, cellular hypertrophy, and enlargement of the gland due to cell multiplication and formation of new acini. This condition, which corresponds to the parenchymatous goitre of human pathology, is a physiological response to conditions adverse to thyroxine synthesis, and is produced by an increased secretion of thyrotropic hormone from the anterior pituitary in response to a lowering of the plasma thyroxine level. The conditions under which this physiological hyperplasia may pass into an irreversible pathological state are not known. Marine and Lenhart (1911) considered that a repeated cycle of hyperplasia and involution took place in the thyroid, and that when the hyperplasia was excessive the involution was incomplete, and eventually led to permanent thyroid enlargement and colloid goitre. An alternative possibility is that the stress of continuous thyroid stimulation renders the cells susceptible to pathological changes which may take a form not predictable by simple theory. Thus both Wegelin (1927) and Hellwig (1935) have reported the occurrence of thyroid adenomata in rats kept on diets which produced a sustained thyroid hyperplasia.

The discovery of the positive goitrogenic effects of the leaves (Chesney, Clawson and Webster, 1928) and the seeds (Hercus and Purves, 1936) of Brassica species, and of the chemical substances related to thiourea (Kennedy, 1942) and the sulphonamides (Astwood, Sullivan, Bissell and Tyslowitz, 1943; MacKenzie and MacKenzie, 1943), provides a convenient method of producing an intense thyroid hyperplasia. The hyperplasia is produced by the same mechanism as that resulting from iodine deficiency, except that the deficient thyroxine output of the thyroid is due to a specific inhibition of thyroxine synthesis instead of a lack of raw material. MacKenzie and MacKenzie (1944) did not find any pathological abnormality produced by prolonged or intermittent feeding of sulphonamides to rats, but their results might have been different if their experiments had been more prolonged. Bielschowsky (1944) considered that allyl-thiourea alone did not produce any neoplastic changes in the rat thyroid, but the hyperplasia induced by it rendered the thyroid susceptible to the tumour-inducing action of simultaneously administered 2-acetyl-amino-fluorene.

The experiments reported in this paper were originally planned to demonstrate

the relative effects of continuous hyperplasia as compared with the repeated cycle of hyperplasia and involution postulated by Marine. The hyperplasia was induced by the administration of the rape seed diet previously described (Kennedy and Purves, 1941). When in the course of these experiments thyroid adenomata were found, further experiments were instituted in an attempt to define the conditions leading to formation of such tumours.

METHODS.

The rats used were of the same strain as have been used in previous experiments. In addition one experiment was carried out on a recent Wistar strain supplied to us by the Animal Research Division of the Agricultural Department of New Zealand. These rats showed certain differences from our own strain, which was originally imported from the Imperial Cancer Research Fund, Mill Hill, England, and has not been maintained by inbreeding.

Four diets were used as follows:

	S1.	S3.	G1.	G2.
Bran	30	30	—	—
Pollard	25	25	—	—
Bone meal	15	15	—	—
Meat meal	15	—	—	—
Skim milk powder	—	—	30	—
Pea meal	—	15	—	30
Maize meal	15	15	25	25
Ground rape seed	—	—	45	45

Iodine was supplied in some experiments by adding potassium iodide to the drinking water. When iodine supplementation is not mentioned, plain tap water was supplied.

Thyroids were dissected out at autopsy, weighed and fixed in formol saline. Sections were cut at 5μ and stained with Harris's haematoxylin and eosin or with Heidenhain's Azan method. In the later experiments the thyroids were cut in serial sections through the whole gland or until adenomata were seen.

RESULTS.

Alternating Diets vs. Continuous Rape Seed Diet.

The rats of groups (*a*) and (*b*) were born from mothers on the diet G1 and had hyperplastic thyroids from birth. At 6 weeks they were transferred to diet S1, which, by virtue of its meat meal content, produces an inactive thyroid gland with colloid storage. Thereafter they were alternated between the two diets with 3-week periods on S1 and 4-week periods on G1.

(*a*) Some rats were killed while on diet S1, after their fifth period of thyroid involution. These animals had thyroids about twice the size of the control groups, but only one-third of the size of such rats which had been continuously on rape seed for the same time. No abnormalities were visible by naked eye inspection. The sections disclosed an involuted gland showing flat epithelium and colloid filled acini.

(*b*) Other rats were killed 4 weeks later while on rape seed for the sixth time, when 41 weeks old. They showed enlarged hyperaemic thyroids. Histologically there was a uniform hypertrophy and hyperplasia as has been previously described (Kennedy and Purves, 1941), but the weight of the glands was less than that of animals kept on diet G1 continuously (group (*c*)).

There was no evidence that the cyclic changes of stimulation and involution had modified the thyroid either morphologically or functionally.

(*c*) A third group of 4 rats, 41 weeks old, was killed at the same time as the rats under (*b*), after having been fed on rape seed diet continuously since they had been weaned from their mothers, who had been on the same diet G1 for several months. Their thyroids were markedly enlarged. The average weight was 70 mg./100 g. body weight. In some glands localized protuberances were superimposed on the general enlargements. The sections disclosed in every gland one or more adenomata. These varied in size from a single acinus up to more than half of the thyroid lobe (i.e. 3 × 5 mm.).

Description of adenomata.

All adenomata had the following characteristics by which they were readily distinguished from the surrounding hyperplastic thyroid tissue: The cells were tall, columnar and much more narrow than the simple hyperplastic cells. The epithelium showed typical infolding and its distal border was irregular. The nuclei were often oval, occupied a basal position, and always stained more intensely than the nuclei of the normal hyperplastic tissue. The abundance of dark staining nuclear chromatin made the adenoma conspicuous within the surrounding tissue. The acini were comparatively large and irregular in outline. The lumen contained pale staining colloid, with considerable debris suspended in it. The larger adenomata showed in addition some layers of fibrous tissue suggesting the formation of a capsule, but this was not the rule. There was evidence of compression of surrounding tissue. The walls of the adenoma acini were stretched and thinned in parts, with signs of rupture and coalescence of adjacent acini. This was variable in different adenomata, some of which were practically converted into a single cystic space filled with colloid, 3–4 mm. in diameter.

Multiple adenomata were common, sometimes two or three appearing in one section. Since serial sections were not always taken through the gland it is not possible to say how many were present. The individual adenomata were approximately spherical, and there was no indication of continuity between the discrete adenomata seen in close proximity. The demarcation from the surrounding tissue was always sharp and definite; nowhere was there any intermediate or transitional zone. Every acinus was either completely normal or frankly adenomatous throughout.

DESCRIPTION OF PLATES.

FIG. 1.—Thyroid of rat after 27 months of rape seed diet. One large, several smaller adenomata. Little normal thyroid tissue remaining. Azan, × 12.

FIG. 2.—17 months' rape seed diet. Adenoma forming nodule projecting from the thyroid surface. Compressed thyroid tissue forming a pseudo-capsule. H. & E., × 24.

FIG. 3.—17 months' rape seed diet. Large adenoma showing cystic spaces filled with dilute colloid. The darker staining of the adenoma tissue is apparent. Azan, × 14.

FIG. 4.—Part of same tumour as fig. 3. Showing mechanism of formation of cystic spaces by rupture of acinar walls. H. & E., × 100.

FIG. 5.—Top left, normal hyperplastic thyroid tissue. Lower right, edge of adenoma, showing the columnar form and darker staining nuclei of the adenoma cells. H. & E., × 650.

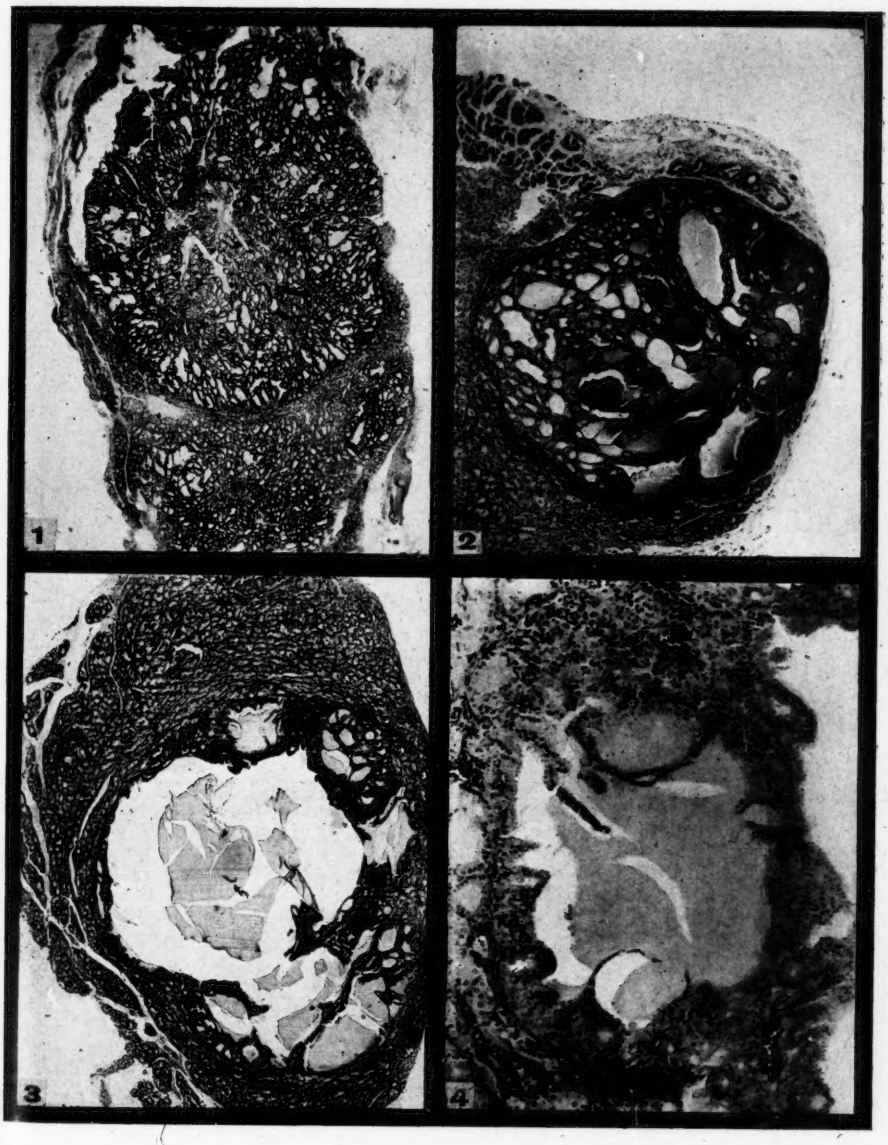
FIG. 6.—Adenoma of fig. 1, showing papillary type of epithelial growth. Azan, × 145.

FIG. 7.—Part of fig. 6. Azan, × 650.

FIG. 8.—From an adenoma, showing undifferentiated type of growth. H. & E., × 650.

FIG. 9.—17 months' rape seed diet, thyroxine injected during last three weeks. The cystic adenoma is filled with dense colloid and the epithelium is flattened. H. & E., × 17.

FIG. 10.—Part of fig. 9, showing the flattened epithelium after thyroxine treatment. H. & E., × 650.



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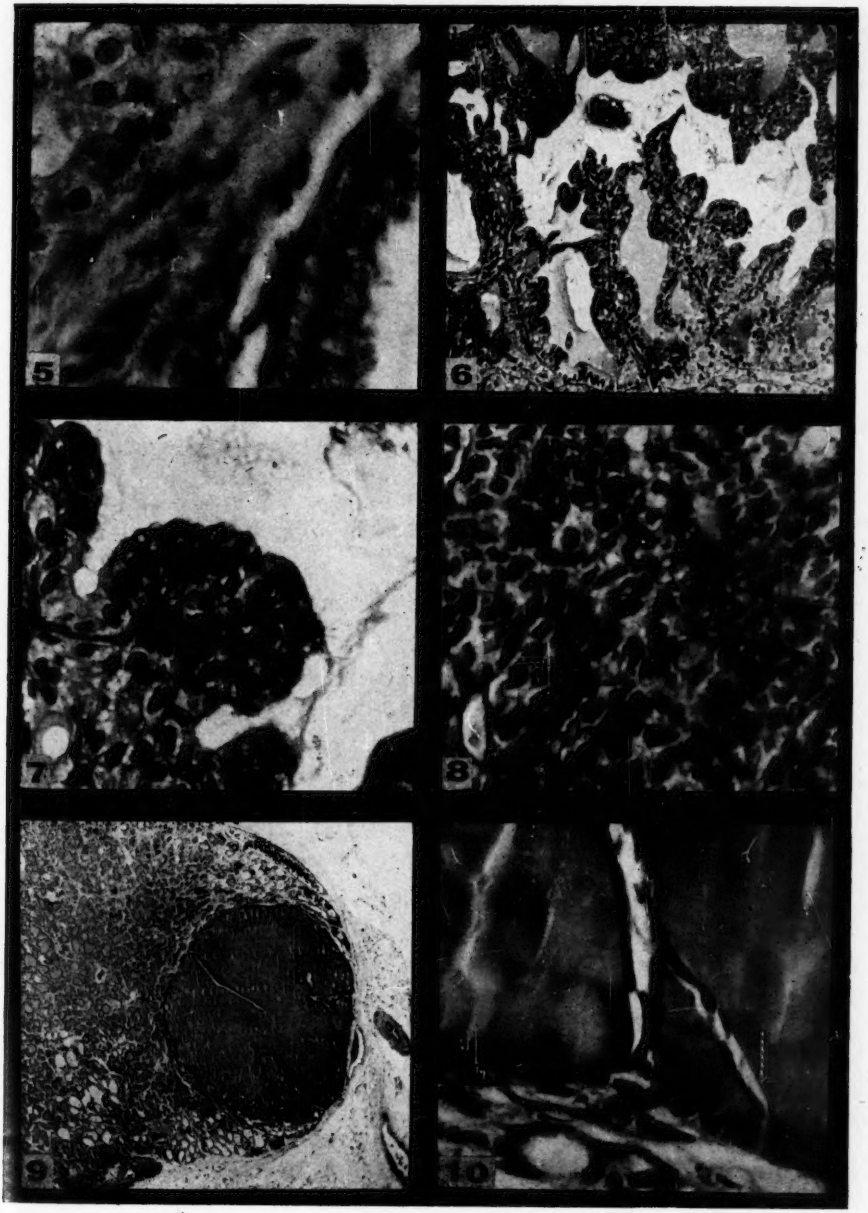

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TABLE I.—*Occurrence of Thyroid Adenomata in Rats.*

Duration of experiment (months).	Number of adenomata found.		
	Iodine rich diet G1.	Iodine poor diet G1.	Iodine poor diet S1.
8	0/4	1/4(a)	0/4
	33·6 mg.(b)	80·0 mg.	12·3 mg.
10	1/4	2/4	0/4
	45·6 mg.	86·9 mg.	21·5 mg.
15–20	11/14	8/10	—
	65·0 mg.	74·6 mg.	—
26–27	4/4	5/5	1(c)/10
	63·2 mg.	—	11·6 mg.
Total	16/26	16/23	1(c)/18

(a) 1/4 means 1 adenoma in 4 animals; (b) average thyroid weight per 100 gm. body weight; (c) thyroid carcinoma.

The Influence of Iodine.

When these adenomata had been found in rats fed on rape seed diet G1 for 10 months and born from mothers which had been on the same diet for some time, an attempt was made to establish some of the conditions which led to the formation of adenomatous growths in the thyroid. A large number of rats of both sexes, 9 weeks old, was changed over from stock diet S1 to rape seed G1 (and later G2). One group was kept in a separate room, and did not get any iodine supplied except that contained in the diet. Potassium iodide was added to the drinking water of the second group to raise the daily intake to 4μg. iodine per rat. A third group was kept on stock diet S1 (and later S3) without any additional iodine. The experiment was continued for 27 months, some rats being killed and examined from time to time. The results are given in Table I. The first adenoma was found after 8 months in a rat in the low iodine group. After 10 months there were 2 rats out of 4 in this group which contained tumours, while in only 1 of 4 rats with added iodine an adenoma was found. The thyroid weight in the latter group was about half of the thyroid weight in the iodine-poor group. In the animals killed after 15–20 months a high proportion of tumours was present. After 26–27 months all thyroids contained adenomata. None of the rats on stock diet with or without added iodine has so far been found to develop a thyroid adenoma, but there was a large thyroid cancer in one rat of the low iodine group which resembled histologically the picture of one type of human thyroid carcinoma. The 32 adenomata found in this series had the same microscopic appearance as has been described for the first series.

Influence of Thyroxine.

The influence of physiological doses of thyroxine (10μg. daily of dl-thyroxine injected subcutaneously for 21 days) has been examined in 8 rats. The adenomata did not disappear, but were transformed under thyroxine influence; the epithelium was completely flat, the acini were filled with well staining, dense colloid, forming large colloid cysts (see Figs. 9 and 10). The non-adenomatous part of the greatly enlarged thyroids reacted similarly, showing general involution.

Susceptibility of Pure Wistar Strain.

A group of 8 rats from a pure Wistar strain was kept on rape seed diet G2 for 12

months. Only one rat of this group showed a thyroid adenoma. The susceptibility of this strain was therefore less than that of the local strain.

Pituitary Changes.

The majority of the pituitaries from rats carrying thyroid adenomata have been examined. Without exception they showed the typical changes described by us for the early stages of stimulation under the influence of Brassicae seed (Griesbach, 1941). The grade of stimulation varied in different animals, but was evidently kept on a high level in most cases for over two years. The basophil cells, which we consider to be responsible for thyrotropin production (Griesbach and Purves, 1945), showed all the signs of high activity, including mitoses. The acidophils were slightly reduced in numbers and granulation. The weight of the pituitaries was generally not increased. All pituitaries of our last group, 5 females nearly 2½ years old, 27 months on rape seed, contained chromophobe adenomata. The histology of these adenomata was identical with those described by Wolfe, Bryan and Wright (1938). The pituitary tissue round the adenomata showed the usual basophil changes. The pituitaries of the group on iodine-poor diets (S1 and S3) showed signs of mild stimulation (increased basophilia).

The pituitaries of the 8 rats receiving 10μg. thyroxine for the last 21 days were found to be normal. The basophils were reduced to low figures and the acidophils were well granulated.

DISCUSSION.

Alternating thyroid hyperplasia and involution, produced in rats by feeding of rape seed diet for 4 weeks and ordinary stock diet for 3 weeks, did not cause any permanent pathological effect during experiments of 41 weeks' duration. The thyroids, after the last period of ordinary diet, were slightly enlarged, but showed no distended acini and no resemblance to colloid goitre. MacKenzie and MacKenzie (1944), working with sulphaguanidine, have obtained similar results. Marine's (1935) hypothesis that the formation of colloid goitre in humans and dogs is due to an alternating cycle of hyperplasia and involution could not be confirmed by these experiments on the rat's thyroid. Moreover, the stress of continued hyperplasia did not lead to any diffuse pathological change in the thyroid tissue, so that these experiments throw no light on the pathogenesis of diffuse colloid goitre.

The discovery of thyroid adenomata in rats which had been under the influence of the rape seed agent from their intrauterine life onwards suggested that the prenatal influence acting at the time of thyroid differentiation might be essential. This is not the case, but the prenatal influence does shorten the time of induction of adenomata.

The induction of the adenomata is considered to be due to the long continued stimulation of the thyroid by the thyrotropic hormone of the pituitary, and not to any specific neoplasia inducing effect of substances in the rape seed. If this is so, adenomata should appear in thyroids maintained in a hyperplastic condition by simple iodine deficiency. This appears to have been the case in the rats observed by Wegelin (1927) and also those of Hellwig (1935), although in the latter's experiments the iodine deficiency was complicated by a calcium excess. Our own experiments with iodine poor diet were unsatisfactory because of fluctuations in the iodine content of the diet, which was not sufficiently controlled.

The administration of iodine to rats on rape seed diet delayed the appearance of adenomata to some extent. This is consistent with the effect of iodine in moderating the hyperplasia produced by the rape seed. An incidence of 100 per cent. was eventually attained in this group, however, and the histological appearances were identical

with those of adenomata produced by the iodine-poor rape seed regime, so that no specific effect is attributable to the iodine.

The effect of thyroxine injections on the histology of the adenomata shows that they are responsive to the influence of the thyrotropic hormone. Thyroxine in the dosage given (10μg. per rat per day) is in excess of the normal daily requirement, and inhibits the secretion of thyrotropic hormone. The adenomata respond promptly to the withdrawal of the thyrotropic hormone with flattening of the epithelium and storage of colloid. The heightened epithelium with papillary proliferation and the thin quality of the colloid as seen in the untreated adenomata are features directly dependent on the increased thyrotropic hormone secretion to which they are exposed, and the atrophic appearance produced by the withdrawal of thyrotropic hormone makes it most unlikely that the adenomata would undergo any proliferation in the absence of the stimulating hormone.

Recently Bielschowsky (1944) has published experiments in which the combined action of a carcinogenic substance (2 acetyl-amino-fluorene) with a goitrogenic substance (allyl-thiourea) produced thyroid tumours in 9 out of 10 rats. Two of these tumours were malignant, while the others, from the illustrations, appear similar to the adenomata described here. Neither of the two substances alone had produced thyroid tumours during the experimental period of almost 10 months, although it is possible that they would have done so if the experiment had been sufficiently prolonged. Bielschowsky thought that the adenomata were no longer under the control of the thyrotropic hormone, basing his opinion on the presence of colloid in some of the nodules. The only test of control by thyrotropic hormone is to observe and compare the behaviour of the adenomata in the presence and absence of thyrotropic hormone. We consider it likely that the adenomata obtained by Bielschowsky were, like ours, responsive to the thyrotropic hormone, and that on this depends the synergistic action of the carcinogen and the goitrogenic agent. The scattered adenoma cells produced by the carcinogen do not grow, or grow too slowly to produce an identifiable tumour, in the absence of the stimulus of considerable amounts of thyrotropic hormone. Experimental evidence on this point could be obtained by following a course of treatment with 2 acetyl-amino-fluorene by a course of treatment with a goitrogenic agent, and comparing with the appropriate controls.

The histological appearance of the pituitaries in rats bearing adenomata shows that a thyroxine deficiency is still present, and therefore that the thyroid cells have not become immune to the action of the goitrogenic agent. Furthermore, the adenomata are not able to elaborate thyroxine while under the influence of the goitrogenic agent. The pituitary adenomata seen in the group of rats nearly 2½ years old are similar to those described by Wolfe, Bryan and Wright (1938) as occurring in old rats of both sexes, with variations of incidence in different strains. We have previously observed these pituiatary adenomata in old rats on rape seed diet but not in old normal rats of our strain, so that in our strain a stimulating influence seems to be necessary in addition to the age effect. There does not seem to be any causal connection between the occurrence of pituitary adenomata and the thyroid adenomata.

SUMMARY.

1. In the rat the repeated cycle of thyroid hyperplasia and involution does not lead to the formation of colloid goitre or thyroid adenomata.

2. Prolonged continuous thyroid hyperplasia produced by the goitrogenic agent in Brassica seeds leads to the formation of thyroid adenomata.

3. The induction of adenomata is not thought to be due to any tumour-forming

agent in the Brassicae, but is considered to be the result of long-continued stimulation by excessive amounts of thyrotropic hormone.

REFERENCES.

ASTWOOD, E. B., SULLIVAN, J., BISSELL, H., AND TYSLOWITZ, R.—(1943) *Endocrinology*, **32**, 210.
BIELSCHOWSKY, F.—(1944) *Brit. J. exp. Path.*, **25**, 90.
CHESNEY, A. M., CLAWSON, T. A., AND WEBSTER, B.—(1928) *Bull. Johns Hopkins Hosp.*, **43**, 261.
GRIESBACH, W. E.—(1941) *Brit. J. exp. Path.*, **22**, 245.
Idem AND PURVES, H. D.—(1945) *Ibid.*,
HELLWIG, C. A.—(1935) *Amer. J. Cancer*, **23**, 550.
HERCUS, C. E., AND PURVES, H. D.—(1936) *J. Hyg., Camb.*, **36**, 182.
KENNEDY, T. H.—(1942) *Nature*, **150**, 233.
Idem AND PURVES, H. D.—(1941) *Brit. J. exp. Path.*, **22**, 241.
MACKENZIE, C. G., AND MACKENZIE, J. B.—(1943) *Endocrinology*, **32**, 185.
MACKENZIE, J. B., AND MACKENZIE, C. G.—(1944) *Bull. Johns Hopkins Hosp.*, **74**, 85.
MARINE, D.—(1935) *J. Amer. med. Assoc.*, **104**, 2334.
Idem AND LENHART, G. H.—(1911) *Arch. intern. Med.*, **7**, 506.
WEGELIN, C.—(1927) *Schweiz. med. Wchnschr.*, **8**, 848.
WOLFE, I. M., BRYAN, W. R., AND WRIGHT, A. W.—(1938) *Amer. J. Cancer*, **34**, 352.

THE REACTION OF PRODUCTS OF INITIAL STAGES OF PEPTIC PROTEOLYSIS OF HUMAN AND HORSE SERUM ALBUMIN WITH ANTISERA TO THE ORIGINAL ALBUMINS.

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IT is known that decomposition products of some antigens can combine with antibodies to the original antigens. Landsteiner *et al.* (1930–31, 1932–33) showed that antibodies to products formed early in the peptic digestion of horse and sheep sera could combine specifically with later products of their peptic digestion, though these products were not precipitable by such combination. Landsteiner (1942) also showed that polypeptides produced by hydrolysis of silk with sulphuric acid can combine specifically but not precipitate with antibodies to silk made soluble by treatment with concentrated HCl.

Little is known of reactions between decomposition products of serum proteins with antibodies to the original proteins. Michaelis (1904), and later Landsteiner and Van der Scheer (1930–31), found that some products of intermediate stages of peptic decomposition of serum proteins do not precipitate with antisera to the original proteins, although, if injected into animals, they can produce antisera which precipitate with them and also with the original proteins. More recently Holiday (1939) reported that some products of initial stages of peptic proteolysis of crystalline horse serum

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albumin did not precipitate with an antiserum to the original albumin, though they still combined with the antibodies and inhibited precipitation of the albumin.

This paper describes a more detailed study of the reactions of some products of initial stages of peptic proteolysis of human and horse serum albumin in precipitation tests with antisera to the original albumins.

MATERIAL AND METHODS.

Serum albumins.—After removing the globulin from human and horse sera by half saturation with $(NH_4)_2SO_4$ and filtering, the serum albumins were precipitated by saturating the filtrates with $(NH_4)_2SO_4$. The precipitates were filtered off, dissolved in H_2O and dialysed.

The *commercial pepsin* used was obtained from the British Drug Houses, Ltd.

Antisera to human and horse serum albumins were produced by injecting 2 ml. of 2 per cent. solutions of the albumins in 0·9 per cent. NaCl intravenously into rabbits twice a week over a period of 5 weeks. The animals were bled 10 days after the last injection.

Serological precipitation tests were made by adding 1 ml. of solutions of tested material to a series of tubes each containing 1 ml. of antiserum solutions so that a range of antigen/antibody ratios was covered. The tubes were placed in a water-bath at 50° C. with columns of fluid half immersed. If no floccules separated after 3 hours' incubation, tests for specific inhibition were made by adding 0·1 ml. of solutions containing unchanged antigen to the tubes. The amount of antigen added always corresponded with optimal proportion for the amount of antiserum in the tubes. Incubation at 50° C. was then continued for another 3 hours. If no floccules separated, this was regarded as specific inhibition.

RESULTS.

The Effect of pH.

One volume of 2 per cent. dialysed human albumin was added to three volumes of 0·033 per cent. pepsin in M/10 citric buffer at various pH values, and the mixtures were incubated at room temperature. Samples were taken at intervals, neutralized and diluted with the amount of saline sufficient to bring the concentration of the original albumin to 0·1 per cent. They were then tested over a range of dilutions against antiserum to human albumin used at 1/30. Inhibition tests were made by adding 0·1 ml. of 0·03 per cent. unchanged human albumin to the tubes where there was no precipitation. The results are given in Table I.

It will be seen that at the three pH values the sequence of changes is similar, though they occur more rapidly at pH 2·0 and 3·0 than at pH 3·5. The protein digest loses its ability to precipitate with the albumin antibodies while retaining its power of specific combination with them, as shown by the ability to inhibit the precipitation of unchanged albumin. As digestion proceeds, however, the ability to inhibit is also lost, and the last products do not react with albumin antibodies in any detectable manner. Increasing the concentration of pepsin merely led to more rapid changes. At pH 4·0 incubation for as long as 24 hours had no effect on the ability of the albumin to precipitate with the antiserum.

Except that the changes took place somewhat more rapidly, similar results were obtained by incubating horse serum with pepsin if the products were tested with the horse albumin antiserum at 1/35.

In the experiments described above tests were made with the antisera at 1/30 and 1/35, dilutions at which the original albumins gave abundant precipitates over a

TABLE I.—*The Effect of Pepsin on the Precipitin Reacti*

Time of incubation with pepsin.	pH 2·0. Dilutions of tested solutions: 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/2.
Control*	—	—	+	+++	+++	+++	++	+	–
5 minutes	i	i	i	i	o	o	o	o	i
10 minutes	i	i	o	o	o	o	o	o	i
20 „	i	i	o	o	o	o	o	o	i
40 „	i	o	o	o	o	o	o	o	i
80 „	o	o	o	o	o	o	o	o	i
24 hours	..	..	..	..	..	..	..	..	..

* Controls incubated without pepsin were neutralized after the same time as the last samples incubated with p

range of antigen/antibody ratios. Some peptic digests, which gave strong inhibition but no precipitation at any antigen/antibody ratio when tested with the antisera at those dilutions, did precipitate when tested with the antisera at 1/10. The floccules, however, appeared much more slowly, were formed over a narrower range of antigen dilutions, were fewer than those formed by unchanged albumins, and the supernatant fluids over the floccules remained opalescent. Thus, the main change in the early stages of digestion is to limit the conditions in which precipitation occurs, and to reduce its rate and extent in the conditions in which it does occur.

All the products of peptic digestion still able to combine with the antibodies to the original albumins are probably contained in the material still precipitable with 2 per cent. CCl_3COOH or with 2/3 saturated $(NH_4)_2SO_4$. This was concluded from the fact that if the material precipitated with 2 per cent. CCl_3COOH from a peptic digest of human or horse serum albumin was dissolved in water, reprecipitated with 2/3 saturated $(NH_4)_2SO_4$, redissolved, neutralized, brought to the original volume, and tested with the antiserum to the original albumin at 1/30 or 1/35, it gave the same "inhibition titre" as the original peptic digest.

After incubating human serum albumin with pepsin for 5 minutes at pH 2·0–3·0 in the conditions described above, 75–90 per cent. of the original material was precipitable by 2 per cent. CCl_3COOH. This amount decreased with increase of incubation time, and the decrease was accompanied by a fall in the "inhibition titre," but the latter proceeded at a much higher rate. It is possible, therefore, that the material precipitable with 2 per cent. CCl_3COOH contained both products able and unable to combine with the antibodies, and the proportion of the latter increased with incubation time. However, even after long periods of incubation, when the digest apparently did not react with the antibodies, it was still possible, by means of precipitation with CCl_3COOH and $(NH_4)_2SO_4$, to concentrate a material which combined with them.

Separation of Fractions from Peptic Digests of Human and Horse Serum Albumin.

When undigested human or horse albumin is precipitated with 2 per cent. CCl_3COOH, redissolved in water, precipitated with 2/3 saturated $(NH_4)_2SO_4$ and resuspended in a volume of water sufficient to bring the concentration of the albumin to 1 per cent., it readily dissolves. The neutralized solution precipitates with the antiserum to the original albumin exactly as does untreated albumin, although the material precipitated with CCl_3COOH differs from the original in that it gives a positive cyanide-nitroprusside test made as described by Harris (1923). On the other hand, when a peptic digest of the albumin, which precipitates with the antiserum to the original albumin diluted 1/10 but not 1/30, is similarly treated, some material does not dissolve after precipi-

n Serum Albumin with its Antiserum. Antiserum at 1/30.

	pH 3·0.								pH 3·5.								
	Dilutions of tested solutions :																
1/64.	1/128.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
++	+	–	+	+++	+++	+++	++	+	–	–	–	+	+++	+++	+++	++	+	–	–
o	o	i	i	i	i	i	i	o	o	–	–	+	++	+++	++	+	–	–	
o	o	i	i	i	i	i	o	o	o	..	..	..	..	..	..	..	..	..	
o	o	i	i	i	i	o	o	o	o	–	–	+	++	+	–	–	–	–	
o	o	i	i	i	o	o	o	o	o	..	..	..	..	..	..	..	..	..	
o	o	i	i	o	o	o	o	o	o	i	i	o	o	o	o	o	o	o	
..	..	..	..	..	..	..	..	..	..	i	i	i	o	o	o	o	o	o	

ubated with p++ ++ + indicate the degree of precipitation.
– indicates no precipitation, if the test for inhibition was not made.
i indicates the presence of inhibition.
o indicates no precipitation and no inhibition.

tation with $(NH_4)_2SO_4$ until the pH is adjusted to about 7·0. When this undissolved material is separated by centrifugation and resuspended in a volume of water equal to that of the fluid from which it was centrifuged, it still remains undissolved until the pH is adjusted to about 7·0.

It was thought possible that fractions of peptic digests differing in solubility after precipitation with CCl_3COOH and $(NH_4)_2SO_4$ might differ in the precipitin test with antisera to the original albumins. To test this, two fractions, one soluble and one insoluble after precipitation with CCl_3COOH and $(NH_4)_2SO_4$, were obtained from peptic digests of human and horse albumin incubated for 5 minutes at pH 2·6 as described above. These will be referred to as the " insoluble " and the " soluble fractions." The fractions were then neutralized, diluted with an amount of saline sufficient to bring the concentration of the original albumin to 0·1 per cent., and tested with the antisera to human albumin diluted 1/30 and to horse albumin diluted 1/35. The results, given in Table II, show that the " insoluble fraction " gave a specific precipitation with the antiserum to the original albumin, although the precipitation was weak, and limited to a narrow range of antigen dilutions. The " soluble fraction," on the other hand, did not precipitate, and gave the phenomenon of specific inhibition. Nitrogen estimations (micro-Kjeldahl) on the precipitates, obtained from the " insoluble " and " soluble fractions " with 2 per cent. CCl_3COOH and washed in 2 per cent. CCl_3COOH, showed that the " insoluble fraction " contained about 10 per cent. and the " soluble fraction " about 80 per cent. of the original nitrogen.

When the " soluble fraction " was tested with the antiserum at 1/10, it precipitated in much the same manner as the original peptic digest. Table III shows the results of testing both the " soluble fraction " and also undigested human serum albumin with the antiserum to human albumin at dilutions of 1/10 and 1/30.

To find possible reasons why the " soluble fraction " precipitates with more concentrated but not with more dilute antiserum, the following experiments were made :

Various dilutions of the " soluble fraction " were mixed with equal volumes of the antiserum at a constant dilution of 1/10 to cover the range of ratios at which precipitation was expected (compare Table III). The mixtures were incubated and then diluted at intervals with twice their volume of saline. If the dilution was made just before precipitation was due to begin, precipitation was prevented even though incubation was subsequently continued for more than a day. Precipitation of unchanged human albumin subsequently added was inhibited, so that the mixtures behaved exactly as they would have done had they been tested with the antiserum diluted 1/30 from the start. Thus dilution after combination between antigen and antibody has taken place is enough to prevent precipitation. By contrast, once precipitation

had occurred with the antiserum at 1/10, dilution had no effect; when the fluids were shaken so as to disperse the floccules, they reappeared within a few minutes. It seems, therefore, that after combination between antigen and antibody, some irreversible changes take place during the formation of a visible precipitate.

Similarly the precipitation of unchanged albumin with its antiserum in the region of antigen excess can be prevented by dilution. Table III shows that precipitation of unchanged human albumin with its antiserum at 1/10 occurred over a wider range of antigen/antibody ratios than with the antiserum at 1/30, so that there was a range of ratios, in the region of antigen excess, where there was precipitation with the antiserum at 1/10 but not at 1/30. If the mixtures with the antiserum at 1/10, corresponding to this range of ratios, were diluted 1/3 with saline just before precipitation would have begun, and the incubation was continued, no floccules appeared. If the dilution was made after flocculation, and the solutions were shaken so that the floccules were dispersed, they reappeared again after a period of incubation.

The compound formed between the "soluble fraction" of the peptic digest and the antibodies to the original albumin at the proportions optimal for precipitation behaves similarly to that formed between unchanged human albumin and the antibodies in the region of antigen excess. These compounds are much more soluble than that formed between unchanged albumin and the antibodies at the proportions optimal for precipitation, and, therefore, they precipitate only partially, and only when their concentration is comparatively high.

Conversion of the "Soluble Fractions" of Peptic Digests of the Albumins into more Precipitable Forms.

The "soluble fractions" of peptic digests of human and horse serum albumin (prepared as in the preceding section) precipitated with the antisera to the original albumins at 1/10 but not at greater dilutions. These fractions, however, could be converted into forms that were again precipitated by the antisera at 1/30 to 1/35 by heating 0·5 per cent. solutions of the preparations at pH 7·0 in the presence of salt. Salts with bivalent cations, e.g. $MgSO_4$, gave better results than those with monovalent cations, e.g. NaCl. The ability of the preparations to precipitate with the antisera was increased only if a considerable opalescence appeared as a result of heating. The following experiment shows an example of such an increase in precipitability:

One sixth volume of 5 per cent. $MgSO_4$ was added to solutions of the "soluble fraction" of peptic digests of both human and horse serum albumin at pH 7·0, and the solutions immersed in a water bath at 100° C. About 1 minute from the moment of immersion, when the fluids were intensely opalescent, heating was interrupted (in order to prevent flocculation) and the solutions were cooled by immersing in cold water. They were then tested against antisera to human and horse albumin at dilutions of 1/30 and 1/35 respectively. It can be seen from Table IV that the "soluble fractions" which previously gave specific inhibition precipitated specifically after they had been heated. The zone of precipitation varied from treatment to treatment. In general, the more intense the opalescence of the heated solutions, the more the zone shifted towards higher antigen concentrations.

Cyanide-nitroprusside Tests and Estimations of Diffusion Coefficient.

Material precipitable with $(NH_4)_2SO_4$ from human albumin incubated with pepsin at pH 2·6 for 5 minutes at room temperature as described above gave a positive cyanide-nitroprusside test. Enough solid $(NH_4)_2SO_4$ was added to the digest to give 2/3 saturation; the precipitate produced was centrifuged down, washed in 2/3

TABLE II.—*The Serological Behaviour of Fractions Separated from Peptic Digests of Human and Horse Serum Albumin*

TABLE II.—*The Serological Behaviour of Fractions Separated from Peptic Digests of Human and Horse Serum Albumin.*

Antigen.	Antiserum to human albumin, dilution 1/30. Dilutions of the antigen.									Antiserum to horse albumin, dilution 1/35. Dilutions of the antigen.								
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
Control of human albumin*	−	−	+	+++	+++	+++	++	+	−	o	o	o	o	o	o	o	o	o
Control of horse albumin*	o	o	o	o	o	o	o	o	o	−	+	+++	+++	+++	+++	++	++	−
Fractions from peptic digests. Soluble after precipitation with $(NH_4)_2SO_4$ prepared from: Human albumin	i	i	i	i	i	o	o	o	o	o	o	o	o	o	o	o	o	o
Fractions from peptic digests. Soluble after precipitation with $(NH_4)_2SO_4$ prepared from: Horse albumin	o	o	o	o	o	o	o	o	o	i	i	i	i	i	i	o	o	o
Fractions from peptic digests. Insoluble after precipitation with $(NH_4)_2SO_4$ prepared from: Human albumin	−	+	++	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Fractions from peptic digests. Insoluble after precipitation with $(NH_4)_2SO_4$ prepared from: Horse albumin	o	o	o	o	o	o	o	o	o	+	++	+	o	o	o	o	o	o

* In the controls undigested albumins were precipitated with 2 per cent. CCl_3COOH and with ammonium sulphate.
The symbols as in Table I.

TABLE III.—*The Effect of Antiserum Concentration on the Serological Behaviour of Human Albumin and of the "Soluble" Fraction of the Peptic Digest of the Albumin.*

Antigen.	Antiserum dilution 1/10. Dilutions of the antigens:									Antiserum dilution 1/30. Dilutions of the antigens:								
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
0·15 per cent. unchanged human serum albumin	+	+++	+++	+++	+++	+++	+++	++	+	−	−	−	++	+++	+++	+++	+++	++
The "soluble" fraction of the peptic digest of human albumin	+	+++	+++	++	+	o	o	o	o	i	i	i	i	i	o	o	o	o

The symbols as in Table I.

TABLE IV.—*The Effect of Heat on the "Soluble Fractions" of Peptic Digests of Human and Horse Serum Albumin.*

Antigen.	Antiserum to human albumin, dilution 1/30. Dilutions of the antigens:									Antiserum to horse albumin, dilution 1/35. Dilutions of the antigens:								
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
Control of human albumin*	−	−	+	+++	+++	+++	++	+	−	o	o	o	o	o	o	o	o	o
Control of horse albumin*	o	o	o	o	o	o	o	o	o	−	+	+++	+++	+++	+++	++	+	−
The "soluble fraction" of the peptic digest from: Human albumin Unheated	i	i	i	i	i	o	o	o	o	o	o	o	o	o	o	o	o	o
The "soluble fraction" of the peptic digest from: Human albumin Heated	+	++	+++	+++	++	+	−	−	−	o	o	o	o	o	o	o	o	o
The "soluble fraction" of the peptic digest from: Horse albumin Unheated	o	o	o	o	o	o	o	o	o	i	i	i	i	i	i	o	o	o
The "soluble fraction" of the peptic digest from: Horse albumin Heated	o	o	o	o	o	o	o	o	o	++	+++	+++	++	+	−	−	−	−

* In the controls undigested albumins were precipitated with 2 per cent. CCl_3COOH and with $(NH_4)_2SO_4$.
The symbols as in Table I.

saturated $(NH_4)_2SO_4$, and suspended in a volume of water sufficient to give about 2 per cent. solution of the material. The solution was then saturated with $(NH_4)_2SO_4$ and the test was made as described by Harris (1923). Undigested human albumin treated in this way gave a negative test.

It was not possible to see whether pepsin causes changes resulting in a positive cyanide-nitroprusside test within the material from which " soluble " and " insoluble fractions " are derived as described above, because precipitation of serum albumin by CCl_3COOH causes such changes.

The intensity of the colour produced in the test with the fraction of peptic digest precipitable with $(NH_4)_2SO_4$ could be increased by heating the material for 10 minutes at 80° C. before testing ; a still more intense colour was given by 1·5 per cent. human albumin incubated with 0·025 per cent. pepsin at pH 2·0 for 24 hours at 37° C., when only a small proportion of material remained which could be precipitated with 2 per cent. CCl_3COOH. This shows that at the initial stage in peptic proteolysis of human albumin, when most of the material is still precipitable by CCl_3COOH, only some of the groups which can be changed by pepsin to give the positive test are so changed.

To see whether the early stage of peptic proteolysis significantly affects the size of albumin particle in the fraction still precipitable by CCl_3COOH, the diffusion rates of undigested albumin and of the " soluble fraction " from a peptic digest were compared by the method of Northrop and Anson (1928–29).

22·5 ml. of 2 per cent. human albumin was mixed with 63 ml. of M/10 citric buffer at pH 2·6, 4·5 ml. of 0·5 per cent. commercial pepsin was added, and the mixture was neutralized after 5 minutes' incubation at room temperature. 10 ml. of water and 25 ml. of 10 per cent. CCl_3COOH were added. The precipitate was dissolved in 12 ml. H_2O and a small amount of undissolved material was centrifuged off. Sufficient solid $(NH_4)_2SO_4$ was added to give 2/3 saturation, the precipitate was centrifuged down and resuspended in 12 ml. H_2O. This suspension was placed in a cellophane sack and dialysed for 5 hours against frequently changed distilled water. Some insoluble material was then removed by centrifugation, and the volume of the fluid was adjusted to 15 ml. with H_2O. This was mixed with 45 ml. of M/15 phosphate buffer at pH 7·0 and 40 ml. of the mixture was used to fill the diffusion cell (by suction through the diaphragm). A funnel-shaped diffusion cell was used, so that the area of its diaphragm of sintered glass was large in relation to its capacity. 40 ml. of the same buffer solution mixed with H_2O in the ratio of 3 to 1 was used as outside fluid. The cell was placed in a constant-temperature room at 16° C., in which the solutions and the cell had been kept for a few hours before the beginning of the experiment. The outside fluid was changed at intervals. The amounts of nitrogen precipitable with 2 per cent. CCl_3COOH in each lot of outside fluid, and also in the first and last inside fluid, were estimated. One fourth volume of 10 per cent. CCl_3COOH was added to each fluid, the precipitate centrifuged down, washed in 2 per cent. CCl_3COOH, and nitrogen determined by the micro-Kjeldahl method.

Undigested human albumin was treated similarly. 5 ml. of 10 per cent. CCl_3COOH were added to 20 ml. of 1·5 per cent. solution of the albumin ; the precipitate was centrifuged down and redissolved in 12 ml. H_2O. Sufficient solid $(NH_4)_2SO_4$ was added to reach 2/3 saturation ; the precipitate was again centrifuged down and resuspended in 12 ml. H_2O. This suspension was dialysed for 5 hours in a cellophane sack against frequently changed distilled water. A small amount of undissolved material was centrifuged off and the volume of the solution was made up to 15 ml. with H_2O. Further procedure was as described above.

From the results, given in Table V, it can be seen that the preparations from the peptic digest of human albumin and from undigested human albumin did not differ

TABLE V.—*Estimation of Diffusion Coefficients.*

Outside fluid removed after:	Time of diffusion (t) (days.)	S.	Q.	$D_{16°}$ (cm.²/day).
The preparation of the peptic digest of human albumin.				
6 hours	0·25	22·50	0·93	0·062
Next 6 hours	0·25	21·57*	0·67	0·046
„ 8½ hours	0·354	20·90*	0·92	0·047
Undigested human albumin.				
8½ hours	0·354	23·50	1·37	0·063
Next 10 hours	0·417	22·13*	1·15	0·047
„ 16½ hours	0·6875	20·98*	1·72	0·047

S = The amount of nitrogen (mg.) in the inside fluid.
Q = „ „ „ passed into the outside fluid.
D = Diffusion coefficient computed from the formula:

$$D = \frac{2{\cdot}3k\ V_1\ V_2}{(V_1 + V_2)t} \log_{10} \frac{SV_2}{SV_2 - (V_1 + V_2)Q},$$

where V_1 is the volume of the inside fluid (40 ml.), V_2 is the volume of the outside fluid (40 ml.), k is the cell constant, which was found to be 0·009 by measuring diffusion of M NaCl at 20° C, for which the diffusion coefficient was taken as 1·11 cm.²/day (see Anson and Northrop, 1936–37).

For derivation of the formula see Northrop and Anson (1928–29).

* Obtained by subtracting from the original the amount that passed to the outside fluid.

in their diffusion rates, the diffusion coefficient of each being 0·047 cm²/day at 16° C. (Diffusion coefficients obtained from estimations of the material which passed into the first lots of outside fluid were different from the value obtained from the subsequent outside fluids, probably because it took some time for the concentration gradient in the membrane and a general state of equilibrium in the system to become established [Northrop and Anson, 1928–29].) The velocity of flow through a capillary was found to be the same for preparations of undigested albumin and of the peptic digest, so it was considered that the solutions tested for diffusion rate did not differ appreciably in viscosity.

Samples of inside and outside fluids from the diffusion experiments were also tested with the antiserum to human albumin. Series of increasing dilutions of the tested solutions with a factor of 1·4 were tested with the antiserum at a constant dilution of 1/30. Within the 50 per cent. approximation thus secured, the distribution of serologically reacting materials between inside and outside fluids coincided with the distribution of nitrogen precipitable with 2 per cent. CCl_3COOH. Undigested albumin was assessed from the beginning and the end of precipitation zones, and from the optimal proportions for precipitation; the peptic digest preparation from the "inhibition titres." It was possible that the solutions tested for diffusion rates were not homogeneous. However, from the agreement between the distribution of serologically active material and of the nitrogen precipitable by CCl_3COOH, it was concluded that the diffusion rates measured probably did correspond with those of serologically active materials.

DISCUSSION.

When digestion of horse or human albumin with pepsin in suitable conditions is sufficiently prolonged, the products do not react in any detectable degree with antibodies to the original albumins. Before this stage is reached the digests cease to precipitate with the antisera in conditions in which unchanged albumins precipitate strongly, although they still combine specifically with the antibodies, for they inhibit precipitation of unchanged albumins subsequently added. The ability to precipitate,

however, is not completely eliminated, for it may occur if the concentration of antiserum is increased sufficiently. Even then it occurs only slowly, and over a narrow zone of antigen/antibody ratios. Two possible interpretations of this can be offered. First the albumin may be changed into products that are still able to combine with antibodies to the original albumin, but are not precipitable by such combination. If this is so, then the precipitates that form in limited conditions would be due to the presence of still unchanged albumin. Alternatively, it may be that such poorly precipitating and inhibiting digests contain no unchanged albumin, but consist of products which form, after combination with antibodies even at optimal proportion, compounds whose solubility is such that precipitation takes place only when their concentration is comparatively high, and even then it is only partial.

The material in the digest capable of combining with antibodies to the original albumin is contained in the fraction precipitable by 2 per cent. CCl_3COOH and 2/3 saturated $(NH_4)_2SO_4$. As this material shows the same diffusion rate as unchanged albumin, it does not appear that the change in serological behaviour arises from a change in molecular size. This is not conclusive, however, for changes in size may have occurred but not been reflected in the diffusion rate because of compensating changes in shape.

A positive cyanide-nitroprusside test given by the fraction precipitated by 2/3 saturated $(NH_4)_2SO_4$ from the peptic digest suggests that certain links in the albumin particles were opened. As the fraction might have contained not only material able to combine with the antibodies, but also some material which had lost this ability, it is possible that the positive cyanide-nitroprusside test was caused entirely by the latter. Reduced precipitability of the material still able to combine with the antibodies, however, shows that certain changes took place in it, and these could be accounted for by assuming breakage of some links. This could be explained by assuming that precipitation of antigen/antibody compounds takes place because, when antigens combine with antibodies, they block in each other the groups on which solubility depends. Breakage of some links in antigen particles, whether it causes splitting of the particles into smaller units or not, would be expected to lead to the opening of new surface areas carrying no specifically combining sites. Combination with the antibodies would thus leave such areas unaffected, and the presence of polar groups in these areas would make the antigen/antibody compound more soluble than that formed between the antibodies and unchanged antigen.

Precipitability of such material by the antibodies to the original albumin can be increased again by heating their solutions in the presence of salt. This is probably a result of the decrease in hydrophilic properties of the material which is known to take place during heat denaturation of proteins (Heymann, 1936; Neurath and Bull, 1936). Antigen/antibody compounds formed by the heated product would consequently be of a lower solubility than those formed by the unheated products.

Although a number of links in albumin particles are probably opened during the initial stage of peptic proteolysis when the material is still precipitable with 2 per cent. CCl_3COOH, enough specifically combining sites seem to remain still unaffected, so that a considerable amount of antibodies to the original albumin can be combined. Further proteolysis, which renders the material soluble in 2 per cent. CCl_3COOH, seems to lead to destruction of the specifically combining sites.

SUMMARY.

Peptic digestion of human or horse albumin in suitable conditions eventually leads to loss of their ability to react in any detectable way with antibodies to the original albumins. Before this stage is reached some products of peptic proteolysis of the

albumins are still able to combine specifically with the antibodies. These products differ from the original albumins in that their precipitability by the antisera to the original albumins is greatly reduced. They do not differ from the original albumins in their diffusion rates. Precipitability of the products by the antisera can be restored by heating solutions of the products in the presence of salt.

REFERENCES.

ANSON, M. L., AND NORTHROP, J. H.—(1936–37) *J. gen. Physiol.*, **20**, 575.
HARRIS, L. J.—(1923) *Proc. Roy. Soc., London*, B, **94**, 426.
HEYMANN, E.—(1936) *Biochem. J.*, **30**, 127.
HOLIDAY, E.—(1939) *Proc. Roy. Soc., London*, A, **170**, 79.
LANDSTEINER, K.—(1942) *J. exp. Med.*, **75**, 269.
Idem AND CHASE, M. W.—(1932–33) *Proc. Soc. exp. Biol.*, **30**, 1413.
Idem AND VAN DER SCHEER, J.—(1930–31) *Ibid.*, **28**, 983.
MICHAELIS, L.—(1904) *Dtsch. med. Wschr.*, **30**, 1240.
NEURATH, H., AND BULL, H. B.—(1936) *J. biol. Chem.*, **115**, 519.
NORTHROP, J. H., AND ANSON, M. L.—(1928–29) *J. gen. Physiol.*, **12**, 543.

CONVERSION OF NON-PRECIPITATING AND INHIBITING PROTEIN COMPLEXES INTO FORMS AGAIN PRECIPITABLE BY THE ANTISERA TO THE ORIGINAL PROTEINS.

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WHEN certain pairs of proteins are heated in salt solutions, they combine during the initial stages of denaturation to form complexes. If one protein is present in the heated mixture in sufficient excess the resulting complex fails to precipitate with antiserum to the minor protein component, though it still combines specifically with its antibodies (Bawden and Kleczkowski, 1941*a*; Kleczkowski, 1941, 1943). This loss of precipitability with specific antiserum, resulting from combination of a protein with another protein into a complex, has now been found to be reversible, and this paper describes the reconversion of the non-precipitating complexes into precipitable forms by incubation with pepsin.

MATERIAL AND METHODS.

The proteins used were purified tomato bushy stunt virus, prepared as described by Bawden and Pirie (1943), and the albumin fractions of human, horse and rabbit serum, prepared as described in the preceding paper (Kleczkowski, 1945*a*). Commercial pepsin from the British Drug Houses, Ltd., was used. The same antisera to human and horse albumin were used as in the work described in the preceding paper. The antiserum to tomato bushy stunt virus was produced by intravenous injections into a

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rabbit of 2 ml. of 0·2 per cent. solution of the purified virus in 0·9 per cent. NaCl. Two injections a week were given over a period of three weeks. Serological precipitin tests were made as described in the preceding paper.

EXPERIMENTAL.

Complexes Formed Between Tomato Bushy Stunt Virus and Serum Albumins.

When a 0·05 per cent. solution of tomato bushy stunt virus in 1·0 per cent. NaCl at pH 7·0 is heated for 10 minutes at 80–82° C., it increases in opalescence and the virus undergoes partial denaturation; infectivity is lost, but the preparation still precipitates with virus antiserum and the protein is still resistant to the proteolytic activity of pepsin. When such a virus solution is heated similarly but in the presence of 1·0 per cent. human serum albumin there is less increase in opalescence, but the solution no longer precipitates with the virus antiserum, although it still precipitates with human albumin antiserum. The preparation still combines, however, with antibodies to the virus, for it specifically inhibits precipitation with virus subsequently added.

The ability of this solution to precipitate with the virus antiserum can be restored by incubation with pepsin. The changes in behaviour of the heated virus-albumin mixture during digestion with pepsin are shown in Table I. One volume of the mixture was added to four volumes of 0·03 per cent. pepsin in M/10 citric buffer at pH 2·6, and then incubated at room temperature. Samples were taken at intervals, neutralized, diluted to correspond to a virus concentration of 0·005 per cent. and tested against antisera to the virus and to human albumin.

Tests with the antiserum to the virus were made by adding 1 ml. of each sample to each of a series of tubes containing 1 ml. of the antiserum at varying dilutions. The test for inhibition was made after 3 hours' incubation at 50° C. by adding 0·1 ml. of 0·05 per cent. virus solution to the tubes in which there was no flocculation.

Tests with the antiserum to human albumin were made by adding 1 ml. of the antiserum at a constant dilution of 1/30 to a series of tubes each containing 1 ml. of the tested samples at varying dilutions. The test for inhibition was made by adding 0·1 ml. of 0·03 per cent. human albumin to the tubes in which there was no flocculation.

The samples were also examined for the amounts of material precipitable with 2 per cent. trichloroacetic acid. One volume of 10 per cent. CCl_3COOH was added to four volumes of each sample. The precipitate was centrifuged down, washed in 2 per cent. CCl_3COOH, and nitrogen was estimated by the micro-Kjeldahl method.

The first result of incubation with pepsin was that the heated virus-albumin mixture ceased to precipitate with human albumin antiserum and specifically inhibited the precipitation of human albumin. The reaction of the preparation with the virus antiserum then still remained unchanged. Further incubation destroyed the ability to inhibit precipitation of human albumin; with the loss of this power the preparation began to precipitate with the virus antiserum. At this stage little of the material in the incubated preparation was precipitated by 2 per cent. CCl_3COOH. Digestion was apparently complete after 48 hours' incubation, when only 6 per cent. of the original nitrogen remained precipitable with 2 per cent. CCl_3COOH. As the nitrogen content of the original virus was 4·5 per cent. of the total, all or almost all of the albumin had then been hydrolysed. Human albumin heated alone was hydrolysed by pepsin at the same rate as albumin heated with the virus.

Similarly, non-precipitating complexes formed by heating the virus with 20 times its amount of rabbit or horse serum albumin were restored to a state precipitating specifically with the virus antiserum. Again inhibition was replaced by precipitation

TABLE I.—*The Effect of Pepsin on the Complex Formed between Tomato Bushy Stunt Virus and Human Serum Albumin.*

Antigen.	Tests with the antiserum to the virus. Dilutions of the antiserum. 1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Tests with the antiserum to human albumin. Dilutions of the antigen. 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	Percentage of N precipitable with 2% CCl_3COOH.
0·005 per cent. unchanged bushy stunt virus	+++	+++	+++	+++	++	+	..	..	..	..	..	..	..	..	..	..
0·1 per cent. unchanged human albumin	..	..	..	..	..	..	−	−	+	+++	+++	++	+	+	−	..
Heated virus-albumin mixture	o	i	i	i	i	i	−	++	+++	+++	+++	++	+	−	−	100
Heated virus-albumin mixture incubated with pepsin. 10 min.	o	i	i	i	i	i	i	i	i	i	o	o	o	o	o	91
Heated virus-albumin mixture incubated with pepsin. 5 hours	o	o	i	i	i	i	i	o	o	o	o	o	o	o	o	58
Heated virus-albumin mixture incubated with pepsin. 24 hours	++	+	o	i	i	i	o	o	o	o	o	o	o	o	o	21
Heated virus-albumin mixture incubated with pepsin. 48 hours	+++	+++	+++	++	+	−	o	o	o	o	o	o	o	o	o	6
Neutralized after: Control*	o	i	i	i	i	i	−	++	+++	+++	+++	++	+	−	−	100

* In the control the heated virus-albumin mixture was incubated at pH 2·6 without pepsin and neutralized after 48 hours.

+++ ++ + indicate the degree of precipitation.
− indicates the absence of precipitation if the test for inhibition was not made.
i indicates the presence of inhibition.
o indicates no precipitation and no inhibition.

as soon as most of the albumin component was hydrolysed beyond the stage of insolubility in 2 per cent. CCl_3COOH.

Incubation of virus-serum albumin complexes for 48 hours at pH 3·5 or at higher pH values, in conditions otherwise similar to those described above, did not result in any change in the reaction with the antiserum to the virus.

Complexes Formed Between Human and Horse Serum Albumin.

In complexes formed between bushy stunt virus and serum albumins one component resists peptic proteolysis whereas the other is hydrolyzed, but pepsin can also be used to convert non-precipitating complexes between two different albumins, both of which are susceptible to peptic proteolysis.

The complex formed between human and horse serum albumin which was not precipitable by the antiserum to human albumin was prepared by heating a solution containing 0·5 per cent. human and 2 per cent. horse serum albumin in 1 per cent. NaCl at pH 7·0 for 5 minutes at 80° C. To restore the ability of this solution to precipitate with the antiserum, the solution was incubated with pepsin at pH 3·5. For comparison a 2·5 per cent. solution of human albumin was heated in similar conditions and then similarly incubated with pepsin. One volume of each solution was added to two volumes of 0·033 per cent. pepsin in M/10 citric buffer at pH 3·5, and the mixtures incubated at room temperature. Samples were taken at intervals, neutralized, diluted to correspond to a 0·1 per cent. concentration of human albumin, and tested with the antisera to human and horse albumin. 1·0 ml. of each tested solution at varying dilutions was added to a series of tubes each containing 1·0 ml. of the antiserum to human albumin at a constant dilution of 1/30 or the antiserum to horse albumin at 1/35. The inhibition was tested by adding 0·1 ml. of 0·03 per cent. human albumin to the tubes where there was no precipitation with the antiserum to human albumin.

From the results shown in Table II it can be seen that after a few hours' incubation with pepsin the solution containing the complex became precipitable with the antiserum to human albumin, although it still remained precipitable with the antiserum to horse albumin. This was accompanied by a considerable decrease in opalescence, presumably due to disaggregation of the aggregates of heat denatured protein particles. With further incubation the precipitation zone with the antiserum to human albumin gradually widened in the direction of higher antigen concentrations, until eventually it corresponded with that given by human albumin which had been heated alone and similarly incubated with pepsin. From this it can be concluded that human albumin is hydrolyzed by pepsin at approximately the same rate irrespective of whether it is treated separately or is part of a complex.

It will also be seen from Table II that, as a result of incubation with pepsin, the zone of precipitation of the complex with the antiserum to horse albumin was shifted even more towards higher antigen concentrations than was the zone of precipitation with the antiserum to human albumin. This indicates that peptic proteolysis of the horse albumin component proceeded at a somewhat higher rate than that of the human albumin component.

A complex between horse and human albumin which did not precipitate with the antiserum to horse albumin was made by heating a solution containing 0·75 per cent. horse albumin and 1·5 per cent. human albumin in 1 per cent. NaCl for 10 minutes at 80° C. at pH 7·0. Peptic digestion also converted this solution into a form that precipitated with the antiserum to horse albumin. The solution was incubated with pepsin, then diluted to correspond to a 0·1 per cent. concentration of horse albumin,

TABLE II.—*The Effect of Pepsin on the Complex between Human and Horse Albumin Non-precipitable by Antiserum to Human Albumin.*

Antigen.		Tests with the antiserum to human albumin. Dilutions of the antigen: 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Tests with the antiserum to horse albumin. Dilutions of the antigen: 1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	1/1024.
Heated mixture of the two albumins		i	i	i	i	i	i	i	o	o	−	−	+	+++	+++	+++	++	+	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after:	½ hour	i	i	i	i	i	i	i	o	o	..	..	..	..	..	..	..	..	..
	1 „	i	i	o	o	o	i	i	o	o	..	..	..	..	..	..	..	..	..
	3 hours	−	−	++	++	+	−	−	−	−	−	−	+	++	++	+	−	−	−
	8 „	−	+	+++	+++	++	+	−	−	−	−	+	++	++	+	−	−	−	−
	24 „	−	+++	+++	+++	++	+	−	−	−	+	++	++	+	−	−	−	−	−
	48 „	+	+++	+++	+++	++	+	−	−	−	++	++	+	−	−	−	−	−	−
	Control*	i	i	i	i	i	i	i	o	o	−	−	+	+++	+++	+++	++	+	−
Human albumin heated alone		−	−	+	+++	+++	+++	++	+	−									
Human albumin heated alone incubated with pepsin. Neutralized after:	3 hours	−	++	+++	+++	+++	++	+	−	−									
	8 „	−	+++	+++	+++	++	+	−	−	−									
	24 „	−	+++	+++	+++	++	+	−	−	−									
	48 „	+	+++	+++	+++	++	+	−	−	−									
	Control*	−	−	+	+++	+++	+++	++	+	−									

* The controls were incubated without pepsin, otherwise in the same conditions. They were neutralized after 48 hours.

The symbols used as in Table I.

and tested with antisera to horse and human albumin as in the preceding experiment. The test for inhibition was made by adding 0·1 ml. of 0·03 per cent. horse albumin to those tubes where there was no precipitation with the antiserum to horse albumin. For comparison a solution containing 2·25 per cent. horse albumin but no human albumin was heated, incubated with pepsin and then tested similarly.

The results, given in Table III, show that after incubation with pepsin for 24 hours the solution containing the complex became precipitable with the antiserum to horse albumin; this precipitation, however, was restricted to a narrow zone of antigen dilutions, was weak and appeared slowly, so that the result was much less spectacular than that obtained in the preceding experiment, when human albumin was the minor component of the complex. This difference can probably be explained by the fact that horse albumin is somewhat more susceptible to peptic proteolysis than is human albumin. The precipitation zone of the complex, after it had been incubated with pepsin, with the antiserum to horse albumin coincided approximately with that given by horse albumin which had been heated alone, and the incubated with pepsin in similar conditions for the same length of time. This indicates that also horse albumin was hydrolyzed by pepsin at a comparable rate, whether it was treated separately or was a part of the complex. It can also be seen from Table III that the intensity of precipitation of the complex with the antiserum to human albumin was less affected by incubation with pepsin than that of horse albumin, which had been heated alone, with its antiserum, and there was less shift of the precipitation zone in the direction of higher antigen excess.

When complexes formed between human and horse albumin were incubated with pepsin for 24 hours at pH 3·5, the amount of material precipitable with 2 per cent. CCl_3COOH fell to about 75 per cent. of the original. After this incubation the complex was converted into a form precipitable with antiserum to either human or horse albumin. When either of the two albumins is incubated with pepsin at pH 2·6, a similar fall in the amount of material precipitable with 2 per cent. CCl_3COOH takes place within a few minutes and, simultaneously, the preparations cease to precipitate with the antisera to the original albumins used at the dilutions as in the experiments described above (compare the preceding paper, Kleczkowski, 1945*a*). This suggests that differences in pH and in substrate concentration not only influence the rate of peptic proteolysis, but also change the process qualitatively.

DISCUSSION.

If the inability of a protein complex to precipitate with the antiserum to its minor component is because the greater part of the complex does not participate in the serological reaction (Kleczkowski, 1945*b*), complete or partial disaggregation of the complex into its constituent parts would be expected to restore precipitability with the antiserum. This would only be expected, however, if the process causing disaggregation does not change the nature of the particles of the minor protein component in such a way that their ability to combine and precipitate with antibodies to the original antigen is destroyed. If pepsin is used to bring about such disaggregation, this condition is easily fulfilled when the minor component of the complex is resistant to peptic proteolysis, but the major component is susceptible. The complex formed between tomato bushy stunt virus and a serum albumin represents such a case. The albumin is hydrolyzed by pepsin in the same way as when treated separately, and the virus component is thus released in the form precipitable by the virus antiserum. In complexes formed between horse and human albumin, on the other hand, when both components are susceptible to peptic proteolysis, they are both hydrolyzed at

TABLE III.—*The Effect of Pepsin on the Complex between Horse and Human Albumin Non-precipitable by Antiserum to Horse Albumin.*

Antigen.	Tests with the antiserum to horse albumin. Dilutions of the antigen: 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Tests with the antiserum to human albumin. Dilutions of the antigen: 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.
Heated mixture of the two albumins	i	i	i	i	i	i	o	o	o	−	−	+	+++	+++	+++	++	+	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 24 hours	+	++	+	−	−	−	−	−	−	+	+++	+++	+++	++	+	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 48 ,,	++	+	−	−	−	−	−	−	−	++	+++	+++	++	+	−	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: Control*	i	i	i	i	i	i	o	o	o	−	−	+	+++	+++	+++	++	+	−
Horse albumin heated alone	−	+	+++	+++	+++	++	+	−	−									
Horse albumin heated alone incubated with pepsin. Neutralized after: 24 hours	+	++	++	+	−	−	−	−	−									
Horse albumin heated alone incubated with pepsin. Neutralized after: 48 ,,	++	++	+	−	−	−	−	−	−									
Horse albumin heated alone incubated with pepsin. Neutralized after: Control*	−	+	+++	+++	+++	++	+	−	−									

* The controls were incubated without pepsin, otherwise in the same conditions. They were neutralized after 48 hours.

The symbols used as in Table I.

approximately the same rate as when treated separately. The solution can be made precipitable by the antiserum to the minor component of the complex, however, by incubation with pepsin in such conditions that hydrolysis proceeds to a point where the complex is sufficiently disaggregated, but most of the protein particles of the minor component are not so changed as to be unable to precipitate with the antiserum to the original antigen.

In order to convert the virus-albumin complex into a form precipitable by the virus antiserum, most of the albumin component had to be hydrolyzed by pepsin at pH 2·6 beyond the stage of precipitability with 2 per cent. CCl_3COOH. A much smaller degree of peptic proteolysis carried out at pH around 3·5 was sufficient to convert the complexes formed between the two serum albumins into forms precipitable by the antisera to their minor protein components. Indeed, if this small extent of proteolysis were not sufficient to convert the complexes formed between the two albumins into such precipitable forms, the conversion would not be possible at all, because further proteolysis would destroy the ability of the components of the complexes to combine and to precipitate with the antibodies to the original albumins. There are indications that peptic proteolysis not only proceeds more slowly at pH around 3·5 than at pH 2·6, but that there are also qualitative differences in the process. It is possible that some links are preferentially broken at certain pH's.

The following reasons can be offered to account for the fact that in the virus-albumin complex much greater proteolysis of the albumin component was necessary to render it precipitable by the virus antiserum than that required to render a human albumin-horse albumin complex precipitable by the antiserum to its minor component. Firstly, in the virus-albumin complex the average number of serologically unspecific particles for one specific particle was much greater than in the human albumin-horse albumin complexes. Secondly, during proteolysis of the virus-albumin complex only the albumin component undergoes changes causing the release of the virus, whereas in the complex formed between the two serum albumins both components undergo changes tending to release each other.

There are a number of viruses with which no precipitin reaction has been obtained, even though they are known to be antigenic as they are neutralized specifically by antisera and some give positive complement-fixation test. No doubt this may often be a result of virus content in the tested solutions being too low to give a visible precipitate. However, it is also possible that some viruses fail to precipitate because they occur in the form of complexes with some unspecific material. If so, treatments that split the complex might release a serologically precipitable component. No example of this is known, but the possibility is emphasized by a recent work on tomato bushy stunt virus. As found in sap expressed from diseased plants this virus is precipitable by its antiserum, but when obtained from the fibre of infected tomato plants after passing through a fine-grinding mill, it is combined into a complex with a chromo-protein (Bawden and Pirie, 1944). This complex does not precipitate with the antiserum to the virus, although it combines specifically with the antibodies. By incubation with commercial trypsin the virus can be released from the complex in a form precipitable with the antiserum.

Non-precipitating complexes prepared by heating protein mixtures can produce antisera specific to only one of their components (Bawden and Kleczkowski, 1941*b*, 1942). In this way it is possible to produce antisera which, although they do not precipitate with the material used for their production, do precipitate with one of the components of the material. It is not certain whether the non-precipitating complexes as such cause production of the antisera, as non-precipitating solutions may contain some material not in the form of such complexes (Kleczkowski, 1945*b*); also, before

acting antigenically, the complexes may be broken *in vivo* into precipitable forms as they can be broken *in vitro* by means of pepsin or trypsin.

SUMMARY.

When tomato bushy stunt virus is heated together with a serum albumin, the virus can combine with the albumin to form a complex that does not precipitate with the antiserum to the virus. The virus, which is resistant to peptic proteolysis, can be recovered in the precipitable form by peptic hydrolysis of the complex. Non-precipitating complexes similarly formed between human and horse serum albumin can, in suitable conditions, be split by means of pepsin into forms again precipitable specifically by antisera, although both components of the complex are susceptible to peptic proteolysis.

REFERENCES.

BAWDEN, F. C., AND KLECZKOWSKI, A.—(1941*a*) *Brit. J. exp. Path.*, **22**, 208.—(1941*b*) *Nature*, **148**, 593.—(1942) *Brit. J. exp. Path.*, **23**, 169.

BAWDEN, F. C., AND PIRIE, N. W.—(1943) *Biochem. J.*, **37**, 66.—(1944) *Brit. J. exp. Path.*, **25**, 68.

KLECZKOWSKI, A.—(1941) *Brit. J. exp. Path.*, **22**, 188.—(1943) *Biochem. J.*, **37**, 30.—(1945*a*) *Brit. J. exp. Path.*, **26**, 24.—(1945*b*) *Ibid.*, **26**, 41.

SPECIFIC PRECIPITATION OF ONE PROTEIN BY ANTISERUM TO ANOTHER.

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BRIEF mention has already been made of the fact that after tomato bushy stunt and tobacco mosaic viruses had been heated together with human serum albumin in suitable conditions, the viruses were precipitated by antiserum to the albumin (Bawden and Kleczkowski, 1941). This was interpreted as evidence that the viruses combine with the albumin during early stages of heat denaturation to form complex aggregates containing virus and albumin. However, only a small number of experiments was made, and the possibility of unspecific adsorption of one component during specific precipitation of the other was not definitely excluded. The present paper presents the results of more detailed experiments, showing that the precipitation is specific, and that the phenomenon is not peculiar to the two plant viruses. After heating together with other proteins, serum albumins and antibodies were specifically precipitated by antisera to the other proteins.

The material and methods of testing used in this work were the same as those used previously (Kleczkowski, 1945).

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EXPERIMENTAL.

The Serological Behaviour of Horse Albumin Heated in an Excess of Human or Rabbit Albumin.

Solutions containing the same amounts of albumins from horse, human and rabbit sera, all heated for 10 minutes at 80° C. in 1 per cent. NaCl solution at pH 7·0, were made by heating the proteins separately and in different combinations. The following scheme shows the details:

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated.

Solution No. 1 } mixed { (*a*) 1 ml. 1·5 per cent. horse alb. + 1 ml. 3 per cent. human alb.
(*b*) 2 ml. 1·5 per cent. rabbit alb.

Solution No. 2 } mixed { (*a*) 1 ml. 1·5 per cent. horse alb. + 1 ml. 3 per cent. rabbit alb.
(*b*) 2 ml. 1·5 per cent. human alb.

Solution No. 3 } mixed { (*a*) 2 ml. 0·75 per cent. horse alb.
(*b*) 2 ml. 1·5 per cent. human alb.
(*c*) 2 ml. 1·5 per cent. rabbit alb.

The total volume of each solution was made up to 12 ml. with 0·9 per cent. NaCl, so that each solution contained 0·125 per cent. of horse albumin, 0·25 per cent. human albumin and 0·25 per cent. rabbit albumin.

The three solutions were tested for their reactions with antisera to horse and human albumin. 1 ml. of each solution at varying dilutions was added to one series of tubes containing 1 ml. of horse albumin antiserum at 1/35, and to another series of tubes containing 1 ml. of human albumin antiserum at 1/15. The tubes were incubated for 3 hours at 50° C., and solutions which did not precipitate with antiserum to horse albumin were then tested for their ability to inhibit the precipitation of untreated horse albumin by adding 0·1 ml. of a 0·03 per cent. solution of horse albumin.

All three solutions precipitated with the antiserum to human albumin. After 3 hours at 50° C. the precipitates were removed by centrifugation, and 0·1 ml. of the antiserum to horse albumin diluted 1/3·5 was added to each supernatant fluid and incubation at 50° C. continued for another 3 hours. Solutions which did not then precipitate were tested for inhibition of precipitation of untreated horse albumin as described above. The results are given in Table I.

Comparing the behaviour of Solutions Nos. 1 and 2 with that of Solution No. 3, in Test A, it can be seen that when horse albumin had been heated *together* with human or rabbit albumin it was converted into a form which did not precipitate with the antiserum to horse albumin and which inhibited the precipitation of unchanged horse

TABLE I.—*Precipitation of Horse Albumin A*

Antigen.	Weights of albumins used for preparation of the solutions (in mg.).			Test (A) with antiserum to horse albumin. Dilutions of antigen:							
	15.	30.	30.	1/1.	1/12.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
Solution No. 1 (horse + human)* + rabbit				i	i	i	i	i	i	i	o
,, No. 2 (horse + rabbit)* + human				i	i	i	i	i	i	i	o
,, No. 3 horse + human + rabbit				−	−	+	+++	+++	++	+	−

+++ ++ + indicate the degree of precipitation.
− indicates no precipitation if the test for inhibition was not made.
i indicates inhibition.
o indicates no precipitation and no inhibition.

albumin. It continued to precipitate after it had been heated alone and then mixed with the other two albumins which had been heated separately.

Precipitating Solutions Nos. 2 and 3 with antiserum to human albumin (Test B) had no effect on their subsequent behaviour when tested with antiserum to horse albumin (Test C), showing that nothing that reacts with the latter had been removed. The behaviour of Solution No. 1, containing horse and human albumin heated together, however, was strongly affected. In all those tubes in which precipitation occurred (except in the tubes where there was antigen excess), inhibition of precipitation of unchanged horse albumin with the antiserum to horse albumin disappeared and in one tube precipitation with the latter antiserum occurred. Clearly the precipitates produced by antiserum to human albumin had removed the material which reacts with the antiserum to horse albumin, and which causes inhibition of precipitation of unchanged horse albumin. Although Solution No. 1 at first did not precipitate with antiserum to horse albumin, it did so after absorption with antiserum to human albumin. This shows that all horse albumin was not converted into a non-precipitable and inhibiting form during the heating, but its precipitation was previously prevented by the presence of the inhibiting form.

The removal of horse albumin converted into the inhibiting form from solution No. 1 by the antiserum to human albumin is not caused by unspecific adsorption. This is suggested by the fact that Solution No. 2, which also contained horse albumin in the inhibiting form produced by heating it together with rabbit albumin, remained unaffected by specific precipitation of human albumin. Also horse albumin heated alone, which remained precipitable with the antiserum to horse albumin (Solution No. 3), remained unaffected by specific precipitation of human albumin. Thus only horse albumin that interacted with human albumin during heating the two *together* can be precipitated by the antiserum to human albumin.

The Serological Behaviour of Human Albumin Heated in an Excess of Horse or Rabbit Albumin.

The following experiment differed from the previous one in that human, instead of horse, albumin was converted into a non-precipitating and inhibiting antigen. This was done by heating the human albumin in the presence of an excess of horse or rabbit albumin ; to do this with human albumin a greater excess of horse or rabbit albumin is necessary than the excess of human or rabbit albumin sufficient to convert horse albumin into an inhibiting form. Except for the changes in proportions of various albumins, and in the combinations in which they had been heated, the experiment was carried out in the same way as the preceding one.

Three solutions, each containing 0·094 per cent. of human albumin, 0·375 per cent.

y Antiserum to Human Albumin.

	Test (B) with antiserum to human albumin.							Test (C) of the supernatant fluids from Test (B) with antiserum to horse albumin.							
	Dilutions of antigen :														
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
	−	+	+++	+++	+++	++	+	i	i	i	+++	o	o	o	o
	−	++	+++	+++	+++	++	+	i	i	i	i	i	i	i	o
	−	++	+++	+++	+++	++	+	−	−	+	+++	+++	++	+	−

* The brackets indicate that the materials were heated together.

horse albumin and 0·375 per cent. rabbit albumin, all heated for 10 minutes at 80° C. in 1 per cent. NaCl at pH 7·0, were prepared. The following scheme shows the differences between the solutions and details of their preparations:

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated:

Solution No. 4 } mixed { (*a*) 1 ml. 0·75 per cent. human alb. + 1 ml. 3 per cent. horse alb.
(*b*) 2 ml. 1·5 per cent. rabbit alb.

Solution No. 5 } mixed { (*a*) 1 ml. 0·75 per cent. human alb. + 1 ml. 3 per cent. rabbit alb.
(*b*) 2 ml. 1·5 per cent. horse alb.

Solution No. 6 } mixed { (*a*) 2 ml. 0·375 per cent. human alb.
(*b*) 2 ml. 1·5 per cent. horse alb.
(*c*) 2 ml. 1·5 per cent. rabbit alb.

The total volume of each solution was made up to 8 ml. with 0·9 per cent. NaCl.

The three solutions were tested with antisera to human and horse albumin as described above. The antiserum to human albumin was used at a constant dilution of 1/30, and the antiserum to horse albumin at 1/10. Tests for inhibition were made by adding 0·1 ml. of 0·03 per cent. human albumin to tubes where there was no precipitation with the antiserum to human albumin. All the solutions precipitated with the antiserum to horse albumin. The precipitates were removed by centrifugation, and 0·1 ml. of human albumin antiserum diluted 1/3 was added to each supernatant fluid. Tests for inhibition of precipitation of unchanged human albumin were made in tubes where there was no precipitation.

The results, given in Table II, show the same phenomena as those of the preceding experiment. Only in Solution No. 4, containing human albumin heated together with horse albumin, did precipitation with the antiserum to horse albumin produce changes in subsequent reaction with the antiserum to human albumin. The product of interaction between human and horse albumin in Solution No. 4, formed when the two were heated together, which inhibited precipitation of unchanged human albumin, was precipitated by the antiserum to horse albumin, except from the tubes where there was antigen excess in the reaction with this antiserum. A similar product of interaction between horse and rabbit albumin in Solution No. 5, formed when the two were heated together, was unaffected by precipitation of horse albumin by its antiserum from the same solution; neither was human albumin in Solution No. 6 precipitable by its antiserum after it had been heated alone and then mixed with horse and rabbit albumin heated separately.

It can also be seen that in three tubes containing Solution No. 4, from which precipitates formed with the antiserum to horse albumin had been removed, there was

TABLE II.—*Precipitation of Human Seru*

Antigen.	Weights of albumins used for preparation of the solutions (in mg.)			Test (A) with antiserum to human albumin. Dilutions of antigen:							
	7·5.	30.	30.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
Solution No. 4 .	(human + horse)* + rabbit		.	i	i	i	i	i	i	i	i
,, No. 5 .	(human + rabbit)* + horse		.	i	i	i	i	i	i	i	i.
,, No 6 .	human + horse + rabbit		.	−	−	+	+++	+++	+++	++	+

precipitation with the antiserum to human albumin. This indicates that this solution contained some of human albumin which did not interact with horse albumin to give the product non-precipitable by the antiserum to human albumin.

The Serological Behaviour of Rabbit Antibodies Heated in an Excess of Human or Horse Albumin.

Similar experiments were made with antibodies using "euglobulin" fraction of the antiserum to tomato bushy stunt virus. This fraction was prepared by adding one volume of a saturated ammonium sulphate solution to two volumes of the antiserum. The resulting precipitate was centrifuged down, washed in 1/3 saturated $(NH_4)_2SO_4$, redissolved in a volume of water corresponding to 3/4 volumes of the original antiserum, dialysed for 24 hours against 0·9 per cent. NaCl, and the total volume was adjusted to that of the original antiserum by adding 0·9 per cent. NaCl. The protein concentration of the solution was 1·0 per cent., and it contained most of the precipitating antibodies of the antiserum.

Three solutions each containing 0·033 per cent. of the "euglobulin" fraction of the antiserum to the virus, 0·166 per cent. of human albumin and 0·166 per cent. of horse albumin, all heated for 10 minutes at 75° C. in 0·9 per cent. NaCl at pH 7·0, were prepared as shown in the following scheme:

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated:

Solution No. 7 } mixed { (*a*) 1 ml. 0·2 per cent. euglob. + 1 ml. 1 per cent. human alb.
(*b*) 2 ml. 0·5 per cent. horse alb.

Solution No. 8 } mixed { (*a*) 1 ml. 0·2 per cent. euglob. + 1 ml. 1 per cent. horse alb.
(*b*) 2 ml. 0·5 per cent. human alb.

Solution No. 9 } mixed { (*a*) 2 ml. 0·1 per cent. euglob.
(*b*) 2 ml. 0·5 per cent. human alb.
(*c*) 2 ml. 0·5 per cent. horse alb.

The volume of each solution was made up to 6 ml. with 0·9 per cent. NaCl.

The effect of treatment of the three solutions with antisera to human and horse albumin was tested. Three 1·5 ml. samples of each solution were measured separately into nine tubes. 1·7 ml. of undiluted antiserum to human albumin was added to one sample of each solution, 1·4 ml. of undiluted antiserum to horse albumin and 0·3 ml. 0·9 per cent. NaCl were added to the second sample, and 1·7 ml. of 0·9 per cent. NaCl to the third. (The amounts of the antisera corresponded with constant antiserum optimal proportions with human or horse albumin, respectively, present in

lbumin by Antiserum to Horse Serum Albumin.

Test (B) with antiserum to horse albumin.									Test (C) of the supernatant fluids from Test (B) with antiserum to human albumin.							
Dilutions of antigen:																
1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1 64.	1/128. 1/256
+	++	+++	+++	+++	+++	++	+	±	i	i	+++	+++	++	o	o	o o
+	++	+++	+++	+++	+++	++	+	±	i	i	i	i	i	i	i	i o
+	++	+++	+++	+++	+++	++	+	±	−	−	+	+++	+++	++	+	− −

Symbols as in Table I.

* The brackets indicate that the materials were heated together.

TABLE III.—*Precipitation of Complexes Formed by Antibodies to Tomato Bushy Stunt Virus with Human and Horse Albumin.*

Test number.	Solution of antibodies to bushy stunt virus.	Weight of materials used for preparation of the solutions (in mg.). 2.	10.	10.	Treatment.	Dilutions of the solutions of antibodies. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.
				Albumin.							
1	Solution No. 7	(euglob. + human)*		+ horse	Incubated with 0·9 per cent. NaCl (control)	i	i	i	i	o	o
2	,, No. 8	(euglob. + horse)*		+ human		i	i	i	i	o	o
3	,, No. 9	euglob. + human		+ horse		+++	+++	+++	++	+	–
4	,, No. 7	(euglob. + human)*		+ horse	Absorbed with antiserum to human albumin	++	+	+	o	o	o
5	,, No. 8	(euglob. + horse)*		+ human		i	i	i	i	o	o
6	,, No. 9	euglob. + human		+ horse		+++	+++	+++	++	+	–
7	,, No. 7	(euglob. + human)*		+ horse	Absorbed with antiserum to horse albumin	i	i	i	i	o	o
8	,, No. 8	(euglob. + horse)*		+ human		+++	++	+	o	o	o
9	,, No. 9	euglob. + human		+ horse		+++	+++	+++	++	+	–

* The brackets indicate that the materials were heated together.

The symbols used as in Table I.

1·5 ml. of each sample.) The mixtures were incubated for 1 hour at 37° C., and then left overnight at 2° C. Precipitates formed in the mixtures which contained the antisera were then removed by centrifugation. Nine different fluids were thus obtained.

Each fluid was then tested for precipitation with the virus and for inhibition of its precipitation by unchanged antiserum to the virus. 1 ml. of varying dilutions of each fluid was added to a series of tubes, each containing 1 ml. of a 0·005 per cent. solution of the virus in 0·9 per cent. NaCl. Inhibition of precipitation of the virus by unchanged virus antiserum was tested by adding 0·1 ml. of the antiserum at a dilution of 1/25 to the tubes where there was no flocculation.

From the results, given in Table III, it can be seen that when the " euglobulin " fraction of the antiserum to the virus had been heated *together* with either human or horse albumin (Solutions Nos. 7 and 8 respectively), antibodies were converted into a form which did not precipitate with the virus, although they combined with it and inhibited its precipitation with unchanged antiserum (Tests Nos. 1 and 2). The antibodies were not converted into such form and were still able to precipitate with the virus when the " euglobulin " fraction was heated alone, and then mixed with human and horse albumin heated separately (Solution No. 9, Test No. 3).

Antibodies converted into the non-precipitating and inhibiting form by heating together with a serum albumin could be precipitated by the antiserum to this particular albumin, but they were not affected by precipitation of another albumin from the same solution. This is shown by the fact that the product causing inhibition of precipitation of the virus could be removed from Solution No. 7, but not from Solution No. 8 by human albumin antiserum (Tests Nos. 4 and 5), and from Solution No. 8 but not from Solution No. 7 by horse albumin antiserum (Tests Nos. 8 and 7). This seems to exclude the possibility that antibodies converted into the non-precipitating form are liable to unspecific adsorption during specific precipitation of a serum albumin. Also antibodies which were not heated together with either of the two albumins, and which were, therefore, able to precipitate with the virus (Solution No. 9), remained unaffected by the precipitation of either albumin by its antiserum (Tests Nos. 6 and 9).

After removal of the inhibiting material both solutions precipitated the virus, although they did so at a lower titre than that given by Solution No. 9, which contained the " euglobulin " fraction heated separately (Tests Nos. 3, 6 and 9). This seems to show that both Solutions Nos. 7 and 8 contained some antibodies which did not interact with the albumins to form products non-precipitable by the virus. The presence of such precipitating antibodies in heated antisera to tomato bushy stunt virus was shown by Bawden and Kleczkowski (1942*b*) by the separation of precipitating fractions from non-precipitating and inhibiting antisera by means of $(HN_4)_2SO_4$ or centrifugation.

DISCUSSION.

Evidence has previously been given that when mixtures of different proteins are heated, heat denatured particles of different kinds of protein can combine to form complex aggregates. For example, when mixtures of certain proteins in salt solutions undergo heat denaturation, interaction products are formed with certain properties different from those of each separate component. Such products are formed at the expense of both protein constituents, and can differ from them in their solubility and precipitability with salts (Kleczkowski, 1941*a*, 1943), in electrophoretic mobility (Van der Scheer *et al.*, 1941), or in serological reactions with specific antigens or antibodies (Kleczkowski, 1941*b* ; Bawden and Kleczkowski, 1941, 1942*a* and *b*). Further evidence for the complex formation is given by the effect of salts on the formation of the interaction products, and by the effect of pepsin on some of such products. The

presence of salts in heated protein mixtures promotes the formation of the interaction products, and the efficiency of different salts in promoting their formation follows Hardy's law, as does their efficiency in causing aggregation of heat denatured protein particles eventually leading to flocculation (Kleczkowski, 1943). If an interaction product is formed by two proteins, one of which is resistant to peptic proteolysis and the other susceptible, certain serological properties of the resistant component can be restored when the other component is hydrolysed by pepsin (Kleczkowski, 1945).

The fact, demonstrated in this work, that a protein heated in the presence of another protein can be specifically precipitated by the antiserum to the other protein, gives the most convincing evidence for the formation of complex aggregates. No alternative explanation of the phenomenon seems to be possible. The serological behaviour of such complex aggregates is most simply explained by assuming that they contain a number of particles of both proteins, and that the ratio of the numbers in an average complex aggregate depends on the ratio of the amounts of the proteins in the heated mixture. The failure of such a complex, composed of two components in unequal quantities, to precipitate with the antiserum to the minor component in spite of combination with the antibodies, is simply explained if we assume that precipitation of antigens by antibodies depends on mutual blocking of the groups determining solubility. As the major component of the complex consists of material which does not participate in the reaction with the antibodies specific for the minor component, only a small proportion of all hydrophilic groups is blocked and the whole complex remains in solution, although its minor component is combined with antibodies. As would be expected, the complex is precipitable by the antiserum to its major component.

A similar interpretation would explain the inability of complexes formed between antibodies and excess serum albumin to precipitate with their specific antigens. Combination of the antibody with its specific antigen would leave hydrophilic groups of the albumin unaffected, and consequently precipitation of the whole complex does not take place. Such antibody-albumin complex is precipitable by the antibodies to the albumin because the albumin is in excess.

As antibody or antigen particles present in such complexes combine with their respective antigens or antibodies, it follows that some at least of their specifically combining groups still remain accessible.

Solutions containing complexes non-precipitable by the antigens or antibodies specific for their minor components also contain certain amounts of precipitable material. This can be demonstrated either by removing the complex, by precipitation with the antiserum specific to its major component, or by fractional precipitation with $(NH_4)_2SO_4$ or by centrifugation (Bawden and Kleczkowski, 1942*b*). In the case of antibodies to " H "-type antigens, e.g. tobacco mosaic virus, the inhibiting activity of the antibody-albumin complex is so small that the precipitating activity of the antibodies which remained precipitable is demonstrable in the presence of the complex (Kleczkowski, 1941*b*).

The presence of serologically precipitable material in the preparations of non-precipitable complexes shows that not all protein particles form similar complex aggregates with particles of another protein. Several explanations of this, not mutually exclusive, can be offered. Firstly some of the protein may remain unaffected by heating. Secondly some protein particles may combine with the particles of the same kind to form aggregates similar to those formed when the protein is heated alone. Thirdly complex aggregates may be formed with ratios of constituents distributed around an average value. In some complex aggregates the ratio of a number of particles of the minor protein component of the heated mixture to that of the major component may thus be sufficiently high to allow their precipitation with antiserum

or antigen specific to the minor component, and to prevent it with antiserum to the major component.

SUMMARY.

Products of interaction between human and horse albumin, formed during heat denaturation of mixtures of the two, could be precipitated by the antisera to either albumin. The product formed in mixtures, where human albumin was in excess, combined with antibodies to horse albumin, but did not precipitate with them. It precipitated with antibodies to human albumin. The converse of this was true when horse albumin was in excess over human albumin in heated mixtures.

Products of interaction formed between antibodies to bushy stunt virus and human or horse serum albumin, formed when mixtures were heated, could combine, but not precipitate, with the virus, although they could be specifically precipitated with antiserum to the albumin.

REFERENCES.

Bawden, F. C., and Kleczkowski, A.—(1941) *Brit. J. exp. Path.*, **22**, 208.—(1942*a*) *Ibid.*, **23**, 169.—(1942*b*) *Ibid.*, **23**, 178.

Kleczkowski, A.—(1941*a*) *Ibid.*, **22**, 188.—(1941*b*) *Ibid.*, **22**, 192.—(1943) *Biochem. J.*, **37**, 30.—(1945) *Brit. J. exp. Path.*, **26**, 33.

Van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. L.—(1941) *J. Immunol.*, **40**, 39.

LAEVULOSE TOLERANCE IN ACUTE HEPATITIS.

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This paper describes certain changes in laevulose tolerance which occur during acute hepatitis. It will be shown that the marked decrease in laevulose tolerance which occurs in many cases of acute hepatitis is probably present in the early stages of all cases of the disease, that the laevulose tolerance curve alters its shape during the progress of the condition, and in most cases becomes normal during the early stages of recovery. Moreover, marked impairment of laevulose tolerance can occur where hyperbilirubinaemia is minimal and clinical jaundice absent. Evidence is presented that such cases of acute hepatitis without jaundice are not necessarily to be regarded as mild cases of the disease. In fact, intensity of jaundice *per se* is no indication of the severity of the underlying liver lesion. It is suggested that impairment of laevulose tolerance may be greatest during the prodromal stages of the disease. The practical application of these findings is discussed in relation to the routine investigation of such cases.

METHODS.

In all the later cases, serum bilirubin was measured either by the method of Malloy and Evelyn (1937), or by a photoelectric modification of the method of Rappaport

and Eichhorn (1943). In the earlier cases and also when the serum bilirubin was below about 1·5 mg. per 100 ml., the Van den Bergh technique described by Harrison (1937) was used. The laevulose tolerance was determined by a modification of the micromethod of Thompson and Wilkinson (1940) using capillary blood. 50 g. of laevulose were given fasting, and blood laevulose was determined beforehand and also ½, 1, 1½ and 2 hours afterwards. In addition, in some cases the oral hippuric acid test was carried out by the original method of Quick (1936), but in the earlier stages of the severer cases of hepatitis the patients often vomited the sodium benzoate, and this is the reason for the infrequency of records of these tests in the early stages of the disease.

MATERIAL.

Forty cases of acute hepatitis of greatly varying degrees of severity have been studied. They are classified according to their aetiology as follows:

		Number of cases.
Epidemic hepatitis		25
Toxic jaundice:	Arsenotherapy	5
	Chloroform	1
	Cinchophen	1
	Toxaemia and septicaemia	1
Homologous serum jaundice		7
		40

Dible, McMichael and Sherlock (1943) have not found any recognizable histological criteria for the differentiation of epidemic hepatitis, arsenotherapy jaundice and homologous serum jaundice, and in the present series, biochemical tests, including determination of laevulose tolerance, of benzoic acid detoxication and of urinary pigment excretion, have also proved valueless in their differentiation. The aetiological groupings will therefore be disregarded and the findings considered as a whole. One case, believed to be an example of acute hepatitis without clinical jaundice, is of considerable interest, and hepatic function was studied in detail. The case-history of this patient is given in the appendix.

RESULTS.

The laevulose tolerance test was performed once only, twice, three and four times on each of 19, 14, 3 and 4 patients respectively. Thus in all, 72 tests have been made. Presentation of all the results would occupy much space, and therefore the table shows the biochemical findings in three typical cases, of which the first had severe jaundice, the second moderate jaundice, and the third was the case referred to above who developed severe prodromal symptoms without actual clinical jaundice.

A wide experience of the laevulose tolerance test as well as a careful scrutiny of the literature suggests that the laevulose tolerance is definitely decreased below normal when the blood laevulose exceeds 15 mg. per 100 ml., *or* when the blood laevulose fails to return to less than 10 mg. per 100 ml. by the end of two hours. It is probable that the 10 mg. per 100 ml. level for the end of two hours is too high, since very rarely indeed in the absence of liver disease does the blood laevulose fail to return to less than 5 mg. per 100 ml. in 2 hours. However, in order to exclude equivocal cases the higher level has been adopted in this communication.

In 25 of the 40 cases (i.e. 62 per cent.) the laevulose tolerance was decreased at

TABLE I.—*Laevulose Tolerance in Acute Hepatitis.*

Case number.	Date.	Blood laevulose in mg. per 100 ml. after 50 g. of laevulose. 0 hr.	½ hr.	1 hr.	1½ hrs.	2 hrs.	Hippuric acid synthesis test (g. hippuric acid excreted).	Total blood bilirubin (mg. per 100 ml).
29 A case with severe jaundice	6.ix.43	..	..	..	..	..	..	13·0
	9.ix.43	..	..	..	..	..	..	16·0
	10.ix.43	0	6	9	11	14	..	..
	11.ix.43	..	..	..	..	..	..	15·8
	19.ix.43	..	..	..	..	..	..	15·0
	20.ix.43	0	13	16	10	7	..	..
	28.ix.43	0	8	16	16	7	..	1·6
	30.ix.43	..	..	..	..	..	3·5	..
	6.x.43	..	..	..	..	..	3·5	..
	8.x.43	0	12	14	4	2	..	..
30 Case with moderate jaundice	23.viii.43	..	..	..	..	..	..	6·2
	26.viii.43	..	..	..	..	..	..	11·6
	27.viii.43	0	8	8	16	26	..	10·0
	30.viii.43	0	24	27	..	10	..	10·4
	2.ix.43	..	..	..	..	..	3·0	..
	3.ix.43	0	20	6	3	2	..	..
	4.ix.43	..	..	..	..	..	..	10·0
	1.x.43	0	7	9	3	1	..	1·2
	5.x.43	..	..	..	..	..	4·8	..
35 A case with severe prodromal symptoms without clinical jaundice	17.ix.43	..	..	..	..	..	..	1·0
	20.ix.43	0	27	22	22	15	..	..
	21.ix.43	..	..	..	..	..	..	0·2
	23.ix.43	0	17	25	13	7	..	..
	24.ix.43	..	..	..	..	..	2·8	..
	30.ix.43	..	..	..	..	..	3·0	..
	1.x.43	0	4	15	15	15	..	..
	12.x.43	0	7	12	12	1	..	..

some stage of the illness, and in most of the cases in which the laevulose tolerance was not decreased, the test was carried out when jaundice had been present for some days. This impairment of laevulose tolerance persisted for a very variable time, which, however, bore no relation to the height of the blood bilirubin or to the duration of the jaundice. In one case the laevulose tolerance was low on the 2nd day of the jaundice, but became normal by the 6th day. In this case the blood bilirubin reached a maximum of 9·7 mg. per 100 ml. 9 days after the onset of jaundice, and persisted above 2·0 mg. per 100 ml. for 15 days. In another case in which serum bilirubin reached 16·5 mg. per 100 ml. and which remained above 2·0 mg. per 100 ml. for approximately 24 days, impairment of laevulose tolerance persisted during the whole of this time. On the other hand, a case in which serum bilirubin reached 29 mg. per 100 ml. showed no impairment of tolerance when it was investigated on the 7th and 22nd days of the jaundice, which lasted approximately 45 days. Conversely, case 35 showed a severe impairment of tolerance, which was present for at least 12 days, although the serum bilirubin was never found to be above 1·0 mg. per 100 ml.

Four cases ultimately progressed to cirrhosis of the liver, and in three of these the laevulose tolerance was diminished at all times when it was investigated. In the

fourth, laevulose tolerance was within the prescribed normal limits when first investigated five months after jaundice began, but four months later the tolerance had decreased. It is perhaps significant that had the less rigid criteria of abnormality been adopted (i.e. failure of the blood laevulose to fall below 5 mg. per 100 ml. in 2 hours), this fourth case would have shown impaired laevulose tolerance.

The form of laevulose tolerance curve was found to vary according to the stage of the disease. Shortly after the jaundice had appeared the blood laevulose often rose steadily throughout the two hours of the test (see curves 1 in Cases 29 and 30). A little later in the disease the blood laevulose reached a maximum about 1 hour after ingestion of the laevulose (see curves 2 and 3 in Cases 29 and 30). Later still, as the patient's condition improved, the blood laevulose never exceeded 15 mg. per 100 ml., but impairment of laevulose tolerance was still present in that the blood laevulose remained above 10 mg. per 100 ml. after 2 hours. This order was, however, not always observed.

In order to compare the relative values of the laevulose tolerance test and the oral hippuric acid synthesis test of Quick as a measure of hepatic function, 49 hippuric acid synthesis tests have been carried out on 33 of the patients and less than the prescribed quantity of hippuric acid has been excreted in 23 cases, i.e. in 70 per cent. of the cases. There has, however, been no absolute parallelism between the two tests. In 17 cases both were positive and in 5 both were negative. In 5 the laevulose tolerance was normal and hippuric acid synthesis diminished, and in the remaining 6 the hippuric acid synthesis normal and the laevulose tolerance diminished. In those cases in which repeated tests were done, the laevulose tolerance appeared to become normal sooner than the hippuric acid (Rennie, 1943).

DISCUSSION.

The Change in Form of the Laevulose Tolerance Curve.

The shape and height of the laevulose tolerance curve is clearly dependent on the amount of laevulose administered, the rate of its absorption from the gut, and the rate at which it is removed from the circulating blood by the liver and non-hepatic tissues. Mann (1934) has clearly shown that laevulose is utilized by the non-hepatic tissues, and some workers therefore condemn the laevulose tolerance test as a measure of hepatic function. It is, however, difficult to see why the non-hepatic utilization of laevulose should be very different from normal in acute hepatitis. Alteration in rate of absorption of laevulose is more probable, especially in view of the gastro-intestinal disturbances which are so frequent in the early stages of the disease, and it is possible that the changes in form and height of the laevulose tolerance curve in acute hepatitis may be due to changes in both glycogenic function of the liver and in rate of absorption from the gut.

The work of Scott (1935), Stewart, Scarborough and Davidson (1938), Herbert and Davison (1938) and Rennie (1943) has shown that in hepatic disease the laevulose tolerance is lower than is encountered in its absence. The more recent work of Higgins, O'Brien, Stewart and Witts (1944), utilizing the sucrose tolerance test, whereby a complicating factor of digestive hydrolysis of sucrose to its constituent monosaccharides is introduced, nevertheless also establishes the fact that the laevulose tolerance of individuals with hepatic disease is much less than that of other subjects.

Soffer (1935), investigating the old form of laevulose tolerance test in which total blood sugar instead of blood laevulose is measured, remarked upon the variability in form of the laevulose tolerance curve, but no special comment has been made on the shape of the specific laevulose tolerance curve until Rae and Wilkinson (1944) reported

their investigations on the effect of burns on liver function. These workers described abnormal curves which were high and flat, and those which showed peaks significantly above 15 mg. per 100 ml. The former type of curve Rae and Wilkinson regarded as indicating delay in removal of laevulose from the blood stream. The results presented here show that both types of curve may be encountered in acute hepatitis, as well as a third type in which the maximum level of blood laevulose is not reached until after 2 hours.

The existence of these types of curve is not without considerable practical importance, since Rennie has modified the laevulose tolerance test by determining blood laevulose only at ½ and 1 hour after ingestion of the sugar. Clearly the slowly rising curve in which the maximum is not reached before 2 hours and the slightly raised flattened (plateau) curve may be missed by such a procedure. Considering the results of Stewart, Scarborough and Davidson (1938), Herbert and Davison (1938) and Herbert (1939), Rennie says: " In curves signifying diminished laevulose tolerance, where blood laevulose did not reach a peak within one hour, in only one instance out of 77 observations did it fail to reach a level higher than in any of the controls." In the present series of 72 observations 3 such exceptions have been encountered, and it is a matter of opinion whether this justifies the determination of blood laevulose at 1½ and 2 hours. Since the micro modification of Thompson and Wilkinson estimates laevulose in *capillary* blood, it is little hardship to the patient for the test to be continued for the additional hour.

Incidence of Impairment of Laevulose Tolerance in Acute Hepatitis.

Rennie found the laevulose tolerance to be decreased in 65 per cent. of 29 cases of acute hepatitis, and the present results are in close agreement with this. However, it must be emphasized that of the 40 cases here described, 19 had the test performed once only, and that test was not necessarily carried out at the height of the disease. Of that 19, 7, i.e. 37 per cent., had impairment of tolerance.

In the remaining 21 cases the test was carried out more than once, and in these an attempt was made to begin the tests as early as possible in the progress of the disease, and the laevulose tolerance was diminished in 18, i.e. 86 per cent. of these cases. This suggests that there might be an impairment of laevulose tolerance in most cases of acute hepatitis, but that the variability is in the time for which this impairment persists. One gains a strong impression that if only the tests were carried out sufficiently early in the disease, all cases of acute hepatitis would show impairment of glycogenic function. It is difficult to be certain of this, since cases are not usually available for investigation until jaundice has been present for some days. A limited experience with the galactose tolerance test performed as described by Maclagan (1940) suggests that it, too, may indicate impairment of glycogenic function in most if not all cases of acute hepatitis if carried out sufficiently early in the disease. Possibly the impairment of glycogenic function is most marked during the prodromal stages. This would be in keeping with the suggestion of Dible, McMichael and Sherlock (1943) that the liver inflammation probably begins with the onset of the prodromal symptoms. In support of this possibility is the fact that there was marked impairment of hepatic function in the case described in this paper, in which no jaundice was present in spite of the development of marked prodromal symptoms.

While it is possible that there is gross impairment of glycogenic function in the early stages of most cases of acute hepatitis, there is great variability in the time for which the impairment persists, and the length of this time bears no relationship to the intensity of the jaundice. It is not difficult to visualize a histological basis for this.

As Dible and his colleagues have shown, there is hepatic cell necrosis and autolysis associated with leucocytic and histiocytic reaction and infiltration. Hepatic regeneration of sufficient extent for normal glycogenic function presumably can occur before there is sufficient restitution of the normal lobular pattern for normal biliary excretion.

Acute Hepatitis without Jaundice.

It has long been recognized that jaundice does not necessarily occur in chronic diseases of the liver, but only recently has it been appreciated that acute hepatitis may occur in the absence of clinical jaundice. Of late the high incidence of acute hepatitis in all its forms has led to the more careful investigation of those cases believed to be in the early stages of the disease, and evidence is now coming to hand that acute hepatitis without jaundice may occur more frequently than has been generally assumed.

Five cases classified by Eppinger (1937) as " icterus catarrhalis sine ictero " suggest that catarrhal jaundice (acute hepatitis) may occur in the absence of jaundice, but a careful scrutiny of the case-histories demonstrates clearly that only one can be regarded as a true acute hepatitis without jaundice. Of the other cases two appear to have experienced some form of food poisoning, the third began as a case apparently of subacute yellow atrophy with jaundice, dying after passing through a non-icteric phase, while the fourth was only seen terminally in coma and was not fully investigated. We are concerned here with those cases of acute hepatitis which develop only the so-called prodromal symptoms, and in which hyperbilirubinaemia is insufficient to cause clinical jaundice. In Eppinger's case of this type, fever, diarrhoea, nausea, headache and fatigue were present. The liver and spleen became palpable and tender, and tests for hepatic function revealed gross impairment. Although urobilinogen was excreted in excess throughout, the patient was never jaundiced and full recovery occurred. Cameron and his co-workers (1943), considering infective hepatitis, were " of the opinion that in some instances hepatitis is present without jaundice occurring at all, although the other clinical symptoms and signs characteristic of the disease are evident." McCallum and Bauer (1944) noted that a " number of those inoculated with icterogenic serum showed evidence of liver damage of insufficient severity to produce jaundice," i.e. they developed symptoms, and showed a rise of serum bilirubin and usually an excess of urobilin in the urine. Damadaran and Hartfall (1944) produced convincing clinical evidence of the occurrence of epidemic hepatitis without jaundice, and their account deserves greater emphasis than it received in their communication.

One of eight officers sharing a billet developed jaundice ; five weeks later four of the remaining seven developed fever, headache, nausea and epigastric pain, but of these four, while three became jaundiced the fourth did not.

As a result of their clinical experience of this and other similar cases, Damadaran and Hartfall concluded that such cases presumably of epidemic hepatitis without jaundice invariably showed an enlarged liver which usually returned to normal size in two weeks, a pronounced relative lymphocytosis, and a temperature which settled in less than a week. Occasionally a trace of bile appeared in the urine, which however usually remained normal throughout, but the significance of this depends on the method they used for detecting bile in urine. It is possible, too, that if Damadaran and Hartfall had tested the urine for urobilin, their cases of epidemic hepatitis without jaundice would have shown an excess of that pigment. Findlay, Martin and Mitchell (1944), in a comprehensive article on hepatitis following yellow fever inoculation, describe seven cases which they regarded as probable examples of the condition without jaundice.

Case 35 developed well-marked prodromal symptoms 59 days after the injection

of the icterogenic serum. Although she was reported as very slightly jaundiced on September 13 a Van den Bergh reaction apparently showed the absence of gross hyperbilirubinaemia. It is fairly certain, then, that if jaundice were really present it must have been very slight. The liver was tender and probably slightly enlarged, since it was just palpable on September 13, but could not be felt six days later. Yet on three of the four occasions when the laevulose tolerance was investigated it showed very marked impairment, and the day after the first of these tests was carried out the blood bilirubin was only 0·2 mg. per 100 ml. Although the hippuric acid synthesis test appeared to show diminished synthesis of hippuric acid, too much reliance could not be put on this test in this case since the sodium benzoate produced much nausea. Measurement of the ether-soluble porphyrin showed that the urinary porphyrin reached a maximum level of about 150μg. per 24 hours, an increase above the normal level of an order known to occur in many cases of acute hepatitis (Nesbitt and Snell, 1942). There is thus good reason to regard this case as one of acute hepatitis without jaundice.

It is possible that acute hepatitis without jaundice occurs more frequently than this brief survey of the literature suggests. These cases are not to be regarded as minor attacks of the disease, since marked impairment of liver function was demonstrated in the case described by Eppinger as well as that presented here. It appears that the condition may be suspected when malaise and gastro-intestinal disturbances are accompanied by an enlarged, tender liver and urobilinuria. In addition, there may be fever, joint pains and headache. Laboratory investigations may show a leucopenia, impairment of glycogenic and other hepatic functions.

Laevulose Tolerance in Cases Proceeding to Chronic Fibrosis.

The persistence of the impairment of laevulose tolerance in three of the four cases which progressed to cirrhosis suggests that the determination of laevulose tolerance might be of value in assessing the prognosis of such cases. However, it must be emphasized that these cases often remain jaundiced to a variable extent throughout their illness, and it is doubtful if determination of laevulose tolerance at this stage would be of practical value, since obstructive jaundice persisting for such a period produces similar alterations in laevulose tolerance. Nor is it to be concluded that impairment of laevulose tolerance is an invariable accompaniment of cirrhosis of the liver. This is very far from being the case, as is well known. The regeneration of liver tissue may be so predominant a factor in cirrhosis that the disease may have progressed very far while laevulose tolerance remains normal. In the past the tendency has been to regard the absence of impairment of glycogenic function in cases of advanced cirrhosis (as judged on clinical grounds) as evidence that such tests are unsatisfactory. The tests are probably adequate, but our interpretation of them has been at fault. A normal laevulose tolerance in a case of advanced cirrhosis with frank circulatory disturbances indicates that liver regeneration has been adequate to maintain relatively normal glycogenic function.

SUMMARY.

1. There is a decrease in laevulose tolerance in the early stages of most, if not all cases of acute hepatitis.

2. The shape of the laevulose tolerance curve varies during the progress of the disease.

3. A case of acute hepatitis without jaundice was found to have marked impairment of laevulose tolerance.

4. It is suggested that there is maximum impairment of glycogenic and other functions of the liver during the prodromal stages of acute hepatitis.

5. Intensity of jaundice appears to be no indication of the severity of the underlying liver lesion.

My thanks are due to all those clinicians who have given me such free access to their cases and to the numerous people who have helped with the blood collections. In particular, Dr. J. L. Livingstone kindly gave me very full facilities for investigating Case 35. Grants from the Central Research Fund of the University of London and from the Research Fund, Diabetic Department, King's College Hospital, enabled special necessary apparatus to be purchased. Finally, particular thanks are due to Miss M. Sandiford and Mr. A. H. Dawson for their able technical assistance.

APPENDIX.

The history of Case 35 was as follows :

On July 3, 1943, M. P—, a female patient aged 43, received an intradermal injection of 0·05 c.c. of serum, which was later found to be icterogenic. (An account of this serum has already been described by Bradley, Loutit and Maunsell (1944).) On August 31, 1943, she became constipated, vomited several times and felt generally unwell. On September 2 the direct Van den Bergh reaction was negative and blood bilirubin was 1·04 mg. per 100 ml. She remained ill with a good deal of nausea until September 13 when she was admitted to a hospital for further investigation. She was then reported to be very slightly jaundiced, the tongue was coated and the throat injected. There was no abnormality of the cardiovascular or respiratory systems. The liver was very tender, and just palpable under the right costal margin. On September 13 the hospital concerned reported the Van den Bergh reaction as " negative," but it has not been possible to find the significance of this statement, and it may be presumed that the reaction indicated no gross hyperbilirubinaemia. On September 16 she still felt very sick, but was not jaundiced ; the urine and stools were normal in colour. The following day (September 17) she was transferred to another hospital for more detailed investigation, as she was believed to be in the prodromal stages of an attack of homologous serum jaundice. She failed to develop jaundice, the liver and spleen were not then palpable, but much epigastric distension persisted until October 2.

Fouchet's test showed that the urine contained no bilirubin ; a slight excess of urobilin was present until September 20, but it is not known how long that excess had been present. The serum bilirubin on September 17 was 1·0 mg. per 100 ml., but had fallen to 0·2 mg. per 100 ml. on September 21. Laevulose tolerance tests were carried out, and as the results show, there was impairment of tolerance on three of the four occasions when the test was performed.

REFERENCES.

Bradley, W. H., Loutit, J. F., and Maunsell, K.—(1944) *Brit. med. J.*, ii, 268.
Cameron, J. D. S., Colville, D. G., Kligler, I. J., and Dales, J. L.—(1943) *Quart. J. Med.*, 12, 139.
Damadaran, K., and Hartfall, S. J.—(1944) *Brit. med. J.*, ii, 587.
Dible, J. H., McMichael, J., and Sherlock, S. P. V.—(1943) *Lancet*, ii, 402.
Eppinger, H.—(1937) ' Die Leberkrankheiten,' Vienna (Julius Springer).
Findlay, G. M., Martin, N. H., and Mitchell, J. P.—(1944) *Lancet*, ii, 340.
Harrison, G. A.—(1937) ' Chemical Methods in Clinical Medicine,' London (Churchill).

HERBERT, F. K.—(1939) *Brit. med. J.*, i, 867.
Idem AND DAVISON, G.—(1938) *Quart. J. Med.*, **7**, 355.
HIGGINS, G., O'BRIEN, J. R. P., STEWART, A., AND WITTS, L. J.—(1944) *Brit. med. J.*, i, 211.
MCCALLUM, F. O., AND BAUER, D. J.—(1944) *Lancet*, i, 622.
MACLAGAN, N. F.—(1940) *Quart. J. Med.*, **9**, 151.
MALLOY, H. T., AND EVELYN, K. A.—(1937) *J. biol. Chem.*, **119**, 481.
MANN, F. C.—(1934) *Ann. intern. Med.*, **8**, 432.
NESBIT, S., AND SNELL, A. M.—(1942) *Arch. intern. Med.*, **69**, 573.
QUICK, A. J.—(1936) *Ibid.*, **57**, 544.
RAE, S. L., AND WILKINSON, A. W.—(1944) *Lancet*, **1**, 332.
RAPPAPORT, F., AND EICHHORN, F.—(1943) *Ibid.*, i, 62.
RENNIE, J. B.—(1943) *Brit. J. exp. Path.*, **24**, 26.
SCOTT, L. D.—(1935) *Ibid.*, **16**, 489.
SOFFER, L. J.—(1935) *Medicine*, **14**, 185.
STEWART, C. P., SCARBOROUGH, H., AND DAVIDSON, G. J. N.—(1938) *Quart. J. Med.*, **7**, 229.
THOMPSON, J. C., AND WILKINSON, A. W.—(1940) *Edin. med. J.*, **47**, 250.

TREATMENT OF EXPERIMENTAL GAS GANGRENE DUE TO *CLOSTRIDIUM WELCHII* WITH PENICILLIN AND ANTITOXIN.

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ON theoretical grounds it would be expected that an established infection due to a toxin-producing anaërobe such as *Cl. welchii* would require the administration of both an antibacterial and an antitoxic agent for its most effective treatment. Penicillin has been shown to be actively bacteriostatic *in vitro* against *Cl. welchii* (Abraham *et al.*, 1941) and to have a significant protective effect in experimental animals if treatment is commenced immediately after infection (McIntosh and Selbie, 1942). We have been able to confirm the bacteriostatic effect with a series of 44 strains of *Cl. welchii*. The main object of the present work was to study the dosage of penicillin and of antitoxin needed to cure *Cl. welchii* infections in guinea-pigs under conditions analogous to those which would be encountered clinically, i.e. with the infection established for several hours before treatment was commenced.

The experiments have shown that when treatment is begun 6 hours after the initiation of infection neither penicillin nor antitoxin alone will prevent death of most of the animals. By the use of a combination of the two it has been possible to protect nearly 100 per cent. of the experimental animals for the 3-day period over which treatment was continued, and to allow indefinite survival for a variable proportion (up to 73 per cent.). The degree of success attained depended largely on the amount of penicillin given.

MATERIALS.

A preliminary examination of available strains of *Cl. welchii* showed that all of 10 recently isolated and 34 strains from old stock cultures were equally sensitive to

penicillin *in vitro*. Growth of these strains was completely inhibited by 0·2 O.U./ml. broth, whereas 0·02 O.U./ml. failed to have any bacteriostatic effect upon them. It therefore did not seem to be necessary to use more than one strain of *Cl. welchii* for the animal experiments.

In most of the experiments a strain of *Cl. welchii*, type A, "NG20a" was used which was originally obtained from a battle casualty in New Guinea. It was forwarded to me through the courtesy of Dr. A. W. Turner and Miss C. E. Eales, of the Council for Scientific and Industrial Research, and had been kept in this laboratory in broth cultures and passaged several times through guinea-pigs. In one experiment a strain of *Cl. welchii* type A, " Gardner," was used. This strain was recently recovered from a human infection which proved fatal within 48 hours of the accident. Both strains were highly pathogenic for guinea-pigs. They were of the invasive type and also good α toxin producers.

Penicillin was obtained from the Commonwealth Serum Laboratories, Melbourne, in the form of the dried sodium salt, sealed in evacuated ampoules. Batch N22–2 (346 O.U./mg.) was used throughout the experiment.

Monovalent gas gangrene antitoxin (Welch A) was also obtained from the Commonwealth Serum Laboratories.

Guinea-pigs of approximately 500 g. weight were used from our own stock and from the Commonwealth Serum Laboratories.

METHODS.

Preliminary work had shown that if reproducible results were to be obtained, infection must be initiated with young actively multiplying bacterial cells free from significant amounts of toxin. The following method of preparing the inoculum was therefore adopted: Three drops of a 16-hour-old " liver piece " broth culture were inoculated into a modified Weinberg's V.F. broth (described as " peptic digest " by Carne, 1940) containing 50 per cent. saline liver extract and 0·1 per cent. ascorbic acid. It was then incubated anaërobically for 3 hours and centrifuged at 4000 r.p.m. for 5 minutes. The supernatant was removed and replaced by an equal volume of broth and the sedimented bacilli were resuspended. This suspension was then diluted 1 in 100 in broth.

Experiments were begun by injecting 0·5 ml. of a 10 per cent. solution of calcium chloride into the muscle tissue of the right thigh of the guinea-pigs to be used. Two hours later 0·25 ml. of the diluted bacterial suspension was inoculated into the prepared sites representing about 1000 lethal doses per animal.

Treatment with penicillin, antitoxin or both was started in most of the experiments 6 hours after the culture had been injected, but in one experiment treatment was started after 9 hours.

The penicillin was dissolved in a potassium phosphate buffer (pH 7·1) and injected intramuscularly in amounts of 0·5 ml. into the left thigh of the animals, i.e. the opposite side to that which received the infection. In all experiments the first dose of penicillin consisted of twice as many units as the subsequent doses. The time intervals between the penicillin injections in the majority of the experiments were 3 hours, but in three experiments were 6 hours.

Treatment was concluded in all experiments after 3 days, i.e. 78 hours after the injection of the culture. The animals were held for observation for a further week.

Treatment with antitoxin was commenced simultaneously with the treatment with penicillin. The antitoxin was injected intramuscularly into the left gluteal muscles in amounts of 0·5 ml. As with penicillin, twice as many units of antitoxin were given with the first injection of each experiment as in the subsequent injections.

The intervals between the antitoxin injections were 8 hours between the first two injections, 12 hours for the two following injections and 24 hours between the final two injections. The total amount of antitoxin in all but one experiment was 1200 units per animal. In one experiment (No. 11) 700 units of antitoxin were injected per animal.

RESULTS OF TREATMENT EXPERIMENTS.

The main series of experiments, in which 12 groups each of 10 or 15 guinea-pigs were used, gave the results tabulated in Tables I and II. Table I demonstrates the relative ineffectiveness of either penicillin or antitoxin alone in contrast to the high rate of survival with the two given in combination at the same dosage.

The control group (1 in Table I) is made up of three groups each of 10 animals, used as individual controls in the various experiments. Three different lots of bacterial inoculum were used and no significant differences were evident amongst these subgroups. The average survival time in this group of 30 untreated animals was 25 hours; the first death took place 18 hours, the last 42 hours after infection.

Groups 2 and 3 given respectively antitoxin alone and penicillin alone both showed an increased survival time, but in each group there was only one survivor when the experiment was concluded at 10 days.

Group 4 animals were given both antitoxin and penicillin in the same total dosage as was given separately in groups 2 and 3. 93 per cent. of these animals survived at the end of treatment and 73 per cent. after 10 days.

Table II includes first the result of an experiment in which five groups (Nos. 5–9) of animals were given increasing doses of penicillin, the dosage of antitoxin being kept uniform. The results show a fairly steady improvement with increasing doses up to a total dosage of 26,000 O.U. of penicillin. The difference between groups 8 and 9 on the one hand, and the three groups (5, 6 and 7) receiving smaller doses, on the other, is statistically significant.

Group 10 gives the result of treatment with 53,000 O.U. of penicillin and 1200 units of Welch antitoxin, the penicillin being given at 6-hourly intervals. Although the difference between the results of this group and those of group 9, in which the same total dosage of penicillin was given at 3-hourly intervals, are statistically not significant, there is some indication that penicillin treatment at 3-hourly intervals is to be preferred.

Group 11 received the same dosage of penicillin as group 10, but only 700 units of antitoxin per animal instead of 1200, and yet there was no appreciable difference in the results. Apparently there is no advantage in using more than 700 units of antitoxin per animal. In addition to the main experimental series an experiment using the same NG20a strain was made to see whether treatment commenced 9 hours after infection could be effective. The scheme of dosage totalling 1200 units of antitoxin and 26,000 O.U. of penicillin was otherwise similar to that of groups 3 and 4. The percentage of survivors at the end of the treatment (given in 3-hourly intervals) was 80 and at the end of the 10th day 47, the average survival time being 73 hours—an indication that even after a delay of 9 hours after the infection, an adequate treatment with penicillin and antitoxin has a significant therapeutic effect.

In another experiment the conditions of group 4 were duplicated except that the penicillin was replaced by sulphadiazine. A total amount of 800 mg. per animal (equivalent to 120 g. per human patient) was used in combination with 1200 units of Welch antitoxin. Treatment was begun 6 hours after the infection with strain NG20a. The sulphadiazine was injected in a powdery suspension in gum arabic subcutaneously into the abdominal region. It was thus injected into the oedematous fluid and tissue

TABLE I.—*Treatment of Guinea-pigs with Penicillin, with Monovalent Antitoxin and with the Two Agents Together. Treatment in All Groups was begun 6 hours after the Infection with* 1000 *L.D. of* Cl. welchii.

Group.	Number of animals.	Time intervals between injections of penicillin. Hours.	Total penicillin per animal. O.U.	Total Welch antitoxin per animal. Units.	Percentage of survivors. End of treatment. 3rd day.	Percentage of survivors. End of treatment. 10th day.	Average survival time at end of treatment.* Hours.	Equivalent human dosage of penicillin. Million O.U.	Statistical significance of results after 3rd day.
1 (controls)	30	—	—	—	0	0	25	—	—
2	15	—	—	1200	40	7	59	—	Different from group 1.
3	15	3	26,000	—	13	7	52	3·6	Not significant from 1 and 2.
4	15	3	26,000	1200	93	73	77	3·6	Different from 1 and 2.

* This includes also the animals which survived to the end of the treatment, their survival time being taken as 73 hours, i.e. the time between infection and last penicillin injection.

TABLE II.—*Delayed Treatment of Guinea-pigs with Varying Doses of Penicillin and Monovalent Antitoxin after Infection with* 1000 *L.D. of* Cl. welchii.

Group.	Number of animals.	Time intervals between injections of penicillin. Hours.	Total penicillin per animal. O.U.	Total Welch antitoxin per animal. Units.	Percentage of survivors. End of treatment. 3rd day.	Percentage of survivors. End of treatment. 10th day.	Average survival time at end of treatment. Hours.	Equivalent human dosage of penicillin. Million O.U.	Statistical significance of results after 3rd day.
5	10	3	3,500	1200	10	10	46	0·5	Not significant from 1 and 2.
6	10	3	7,000	1200	40	10	60	1·0	Different from 1, not from 2.
7	10	3	14,000	1200	40	30	68	2·0	Different from 1, not from 2.
8*	15	3	26,000	1200	93	73	77	3·6	Different from 1, 2 and 7.
9	15	3	53,000	1200	100	73	78	7·4	Different from 1, 2 and 7.
10	15	6	53,000	1200	93	40	77	7·4	Different from 2, not from 9.
11	10	6	53,000	700	100	50	78	7·4	Different from 2, not from 10.

* This group is identical with group 4 in Table I; it has been repeated in this table for the sake of comparison. Groups 1 and 2 of Table I serve as control groups.

in which the infection was spreading. There was a survival rate of 66 per cent. at the end of the 3rd day and 27 per cent. at the end of the 10th day. The blood level of sulphadiazine 24 hours after the end of the treatment was 9·2 mg./100 ml.

One group had been infected with a different Welch strain than the previous groups, strain " Gardner." In this group recurrence of the infection appeared in the majority of the animals after the treatment (given in 6-hourly intervals) had been discontinued. The animals died approximately 36 hours after the end of the treatment, with an infection extending over the whole ventral surface of the trunk. The percentage of survivors at the end of the treatment was 93, and at the end of the 10th day 27.

The statistical evaluation of the experiments was carried out by Mr. E. A. Cornish, of the Biometric Section of the Council for Scientific and Industrial Research, Melbourne. The analysis of the results after three days is shown in the last columns of Tables I and II. Wherever the probability of the observed difference being due to chance alone was more than 0·02, it was regarded as not significant. In the great majority of the groups the probabilities were of about the same order when the results at either the 3rd or the 10th day were compared. However, the result in group 2 differed significantly from that of group 1 at the 3rd day, but not at the 10th day. This also applied to group 6 as compared with group 1. The result of the group which was treated with sulphadiazine and antitoxin was not significantly different from group 2 which received antitoxin treatment only, whereas there was a statistically significant difference between the sulphadiazine treated group and the group treated with penicillin and antitoxin (group 9) in favour of the latter.

PATHOLOGY.

Some guinea-pigs were infected by the standard technique, but were left untreated and killed 6 hours after the culture had been injected. The infection had at that time spread widely into the tissues of the muscle injected and had already penetrated into the surrounding subcutaneous tissues. Discoloration and small haemorrhages could be observed in the affected muscle, and gas formation had taken place to a considerable extent inside the muscle and to some degree in the subcutaneous tissue. Untreated guinea-pigs, killed 18 hours after the infection, showed very severe digestion of the thigh and buttock muscle tissues with infection spreading into the abdominal muscle and subcutaneous tissue right up to the thorax, with oedematous fluid and much gas formation. A number of guinea-pigs treated 3-hourly with penicillin (5000 O.U.) and antitoxin (600 units) were killed 18 hours after the infection to ascertain the tissue damage and progress of the infection at that stage. The thigh muscle into which the culture had been given also showed severe destruction and discoloration due to the infection, which also extended to the abdominal tissues. There was, however, a very marked difference between the infection of the untreated and treated animals. In the untreated guinea-pigs the infection had spread over both sides of the whole abdomen and lower thorax, whereas in the treated guinea-pigs the spreading was confined to the lower abdominal region and only to the side on which the infection had been given. There was also much less gas formation in the treated animals. Animals infected with strain NG20a and treated with penicillin and antitoxin which died between the end of the treatment and the 10th day showed on post mortem no fresh flaring up of the infection. In the majority of the animals a fibroblastic wall had been built up around the area of infection. The same was the case with animals which survived the infection for more than 10 days. A microscopic section of a thigh muscle taken from a treated guinea-pig, which was killed 14 days after the infection, is shown in Fig. 1

Table I serve as control groups.

(Gram Weigert strain). Gram-positive bacilli in long filaments indicating an inhibition of cell division and unusually long spores could still be seen in these sections.

A number of swabs were taken from the infected muscle tissues of treated guinea-pigs, which were killed 18 days after the infection. From all swabs pure cultures of *Cl. welchii* were obtained. Two of these cultures were tested for their sensitivity to penicillin. Neither showed any loss of sensitivity to penicillin as compared with the original strain. These cultures were also tested for their pathogenicity against guinea-pigs, and there was no obvious loss as compared with the original culture.

TESTS FOR TOXICITY OF PENICILLIN.

As penicillin is known to be toxic for guinea-pigs when used in very large doses given over a period of several days (Hamre and Rake, 1943), preliminary tests were carried out on these animals with the penicillin from the same batch as was used in all experiments described in this paper.

It was found that this batch of penicillin was slightly toxic only in the largest dosage used in the treatment experiments, that is, 53,000 O.U. if given in 3-hourly injections for 3 days. Of 10 guinea-pigs receiving the dosage only 1 died. Another group of 5 animals was injected over a period of 4 days with a total of 69,000 O.U. of the same batch of penicillin; 3 of these died between the 5th and 7th day.

Another batch of penicillin containing 308 O.U./mg. was also tested for its toxicity in guinea-pigs. Ten animals were injected with 37,000 O.U. in 4 days. In this group the injections were given at 6-hourly intervals; 5 of these animals died.

A number of sections were made by Major F. R. Magarey for microscopical examination of the organs of these guinea-pigs and the diagnosis kindly forwarded to me. The thigh muscle which was injected with the penicillin showed patches of coagulative necrosis, surrounded by a zone of fibroblastic proliferation with many multinucleated giant cells of the foreign body type. The arteries of this muscle showed a peculiar vacuolation and hypertrophy of the endothelium (Fig. 2). No obvious pathological changes were observed in other organs.

In view of the suggestion in Florey and Florey's work (1943) that a rise in the blood urea occurred in some patients under penicillin treatment, blood urea estimations were carried out in some of these guinea-pigs before and after administration of penicillin. Before treatment the values averaged about 60 mg./100 ml. The values between the 3rd and 7th day showed a variation between 70 and 112 mg./100 ml. There was no obvious correlation between the urea content of the blood and whether or not the animal survived.

SUMMARY AND CONCLUSIONS.

The importance of combining antitoxin with penicillin therapy of experimental *Cl. welchii* infections in guinea-pigs has been clearly demonstrated by these experiments. The dosage required over a 3-day period corresponds approximately to a total dosage of 3 to 4 million Oxford units of penicillin and 150,000 units of antitoxin for an adult human patient.

I am greatly indebted to Dr. S. E. Williams, Sgt. M. Moore and Cpl. P. Hamilton for their assistance in the work, to Miss B. Splatt of the Royal Melbourne Hospital for the blood urea estimations, to Major F. R. Magarey for carrying out the pathological examinations, to Mr. E. A. Cornish for the statistical evaluations, to Mr. N. Southern of the C.S.I.R. for preparing the photographs, and to Col. E. V. Keogh and Dr. F. M. Burnet for constant helpful advice and interest in this work. The work was supported by a grant from the Commonwealth Government Scientific Liaison Bureau.

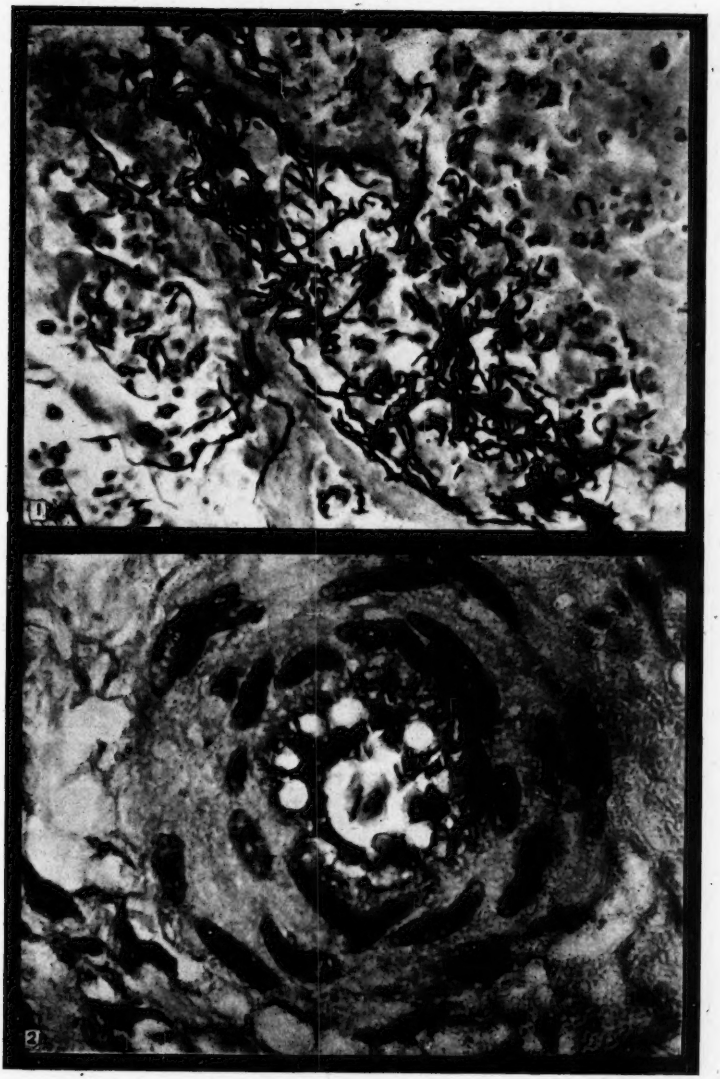

FIG. 1.—Section of thigh muscle of a guinea-pig which was infected with *Cl. welchii* and treated with penicillin and antitoxin. Excised 11 days after conclusion of treatment. Gram-positive bacilli in long filaments can be seen, indicating inhibition of cell division (Gram Weigert stain). × 750.

FIG. 2.—Section of artery from guinea-pig thigh muscle, which was injected with 37,000 O.U. of penicillin. Note vacuolation of the endothelium. × 800.

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REFERENCES.

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A., AND FLOREY, H. W.—(1941) *Lancet*, ii, 177.
CARNE, H. R.—(1940) *J. Path. Bact.*, **51**, 199.
FLOREY, M. E., AND FLOREY, H. W.—(1943) *Lancet*, i, 387.
HAMRE, D. M., RAKE, G., MCKEE, C. M., AND MACPHILLAMY, H. B.—(1943) *Amer. J. med. Sci.*, **206**, 642.
MCINTOSH, J., AND SELBIE, F. R.—(1942) *Lancet*, ii, 750.

ACCELERATION BY MEANS OF PROLONGED MECHANICAL IRRITATION OF CARCINOGENESIS IN THE SKIN OF MICE PAINTED WITH 1 : 2 : 5 : 6-DIBENZANTHRACENE.

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TWENTY years ago Deelman claimed to have observed the appearance of malignant tumours in wounds placed in the skin of mice which had been subjected to tar painting (Deelman, 1923–24). This observation led to many experiments on the relationship of trauma to carcinogenesis, though the results, when reviewed, appeared to be conflicting in the extreme (Eggers, 1932; Seelig and Cooper, 1933).

In many instances it is now apparent why decisive results were not obtained in these early experiments. Most of the investigations were carried out on small numbers of mice of mixed sexes and various strains. The tar was often of doubtful potency, and either the carcinogen or its solvent was apt to cause non-specific irritation, which can be avoided with appropriate doses of the pure hydrocarbons, provided they are not themselves too powerful to mask slight co-carcinogenic or anti-carcinogenic effects, and are applied at intervals so spaced that non-specific irritation to the skin is minimized. For this purpose acetone forms a more suitable solvent for the hydrocarbon than benzene (Orr, 1938).

Recently, two satisfactory experiments have been reported in which repeated trauma by means of heat markedly accelerated the appearance of tumours in the skin of mice which had received preliminary treatment with a carcinogenic hydrocarbon (des Ligneris, 1940; Lauridsen and Eggers, 1943). Des Ligneris, for example, states that if the thermal irritation is applied " at a time when only some of the animals have reached the cancer stage, it accelerates tumour production and cancerous change almost as effectively as the continuation of the specific treatment."

Such results stand in sharp contrast to the findings in some of the early experiments to which reference has already been made. Ludford, for example, who used small groups of animals, found that a weekly application of sandpaper to the skin of mice which had previously been tarred reduced the likelihood of subsequent tumour formation (Ludford, 1929). This worker agreed, however, that such a reduction in

the tumour yield might have been due to the removal by the sandpaper of those cells which had already come under the influence of the tar. It was also noted that this form of irritation led to the development of subepithelial fibrosis at the site of injury.

The present experiment was therefore designed to inquire further into the effect of mechanical, as opposed to thermal, irritation of the skin of mice which had been treated with a suboptimal dose of a carcinogenic hydrocarbon.

EXPERIMENT.

Two hundred inbred male mice of the Medical Research Council strain, four to eight weeks old, were painted once weekly for 16 weeks with a 0·3 per cent. solution of 1 : 2 : 5 : 6-dibenzanthracene dissolved in acetone. Fifteen mice died during this period and no ulceration or papilloma formation occurred.

At the end of this time the mice were divided into two groups of 93 and 92 mice. The painted areas in the animals of Group "A" (93 mice) were then brushed twice weekly with a small brush of stiff bristle—a form of mechanical irritation which preliminary experiments had shown to be unassociated with surface ulceration. Group "B" (92 mice) remained as a control.

After 33 weeks of this treatment a clear result was obtained ; and as the numbers of mice which had died without tumour were not unduly high (17 mice in Group " A " ; 21 mice in Group " B "), it was decided to carry the experiment a stage further by stopping the mechanical irritation in Group " A " and by again painting both groups with the carcinogen. On this occasion the 0·3 per cent. solution of dibenzanthracene was applied twice weekly for a further sixteen weeks. The whole experiment extended over 75 weeks.

Sections of skin were taken from mice which died, and further sections were later obtained from those mice in both groups which survived the full period of the investigation. In making an assessment of the tumour yield only those papillomas which remained palpable for at least six successive weeks were taken into account, and for this reason one animal which developed a papilloma during the last weeks of the experiment was allowed to survive until this period had elapsed. Tumours were recorded as malignant when they became large, fixed or ulcerated, and each diagnosis was later reviewed in the light of histological examination. The results are set out in the form of a graph.

RESULTS AND CONCLUSIONS.

Papillomas began to appear in both groups of mice within two weeks of cessation of painting ; and for the next six weeks there was no significant difference between the two groups. Thereafter the numbers of both papillomas and carcinomas were significantly higher in the brushed group " A " than in the controls " B." At the 49th week 55 mice in Group " A " had borne tumours, of which 13 were malignant ; while in Group " B " the number of papillomas was 36, of which only 4 had undergone malignant transformation. The additional factor of the mechanical trauma in Group " A " must be held responsible for this difference in tumour emergence in the two groups.

On the resumption of painting, fresh papillomas appeared in both groups of mice, while several pre-existing papillomas which had become stationary took on rapid and invasive growth. Despite the fact that there were now relatively more mice available for papilloma formation in Group " B," the tumour yield in this group 10 weeks after cessation of the second period of painting still lagged behind that of Group " A."

Indeed, at the 75th week the number of tumours in the controls had not quite reached the level of tumour production which the brushed group had previously attained at the 49th week, i.e. at the time when the second period of painting was begun. At the end of the experiment the total number of carcinomas (9) in the control group was also less than the number of carcinomas (13) in the brushed group at the 49th week. The brushed group finally yielded a total of 21 carcinomas. The failure to obtain more carcinomas in the controls during and after the second period of painting can

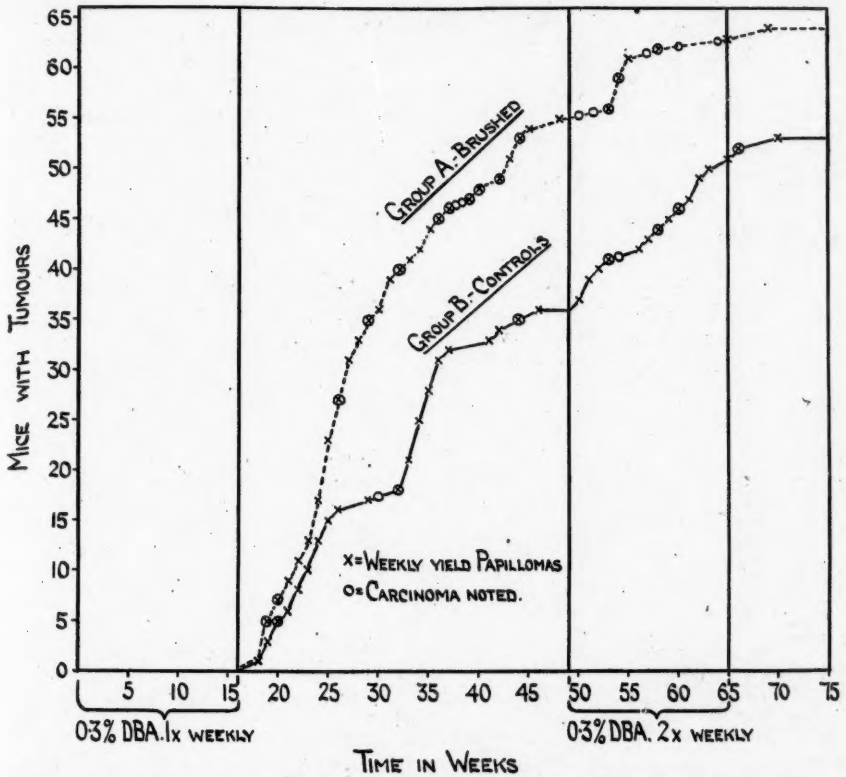

FIG. 1.—Graph showing the appearance of papilloma and carcinoma during the experiment.

be partly accounted for by the rapid death rate which took place from the 60th week onward in both groups of mice. Nevertheless there would appear to be no doubt that, under the conditions of the experiment, mechanical irritation by brushing following a suboptimal period of painting with dibenzanthracene markedly favoured subsequent tumour formation. This finding is thus in agreement with the results obtained by des Ligneris and by Lauridsen and Eggers in their experiments with thermal trauma inflicted under comparable conditions.

Finally, histological examination of sections of skin from the mice in Group "A" failed to show more marked subepithelial fibrosis than in the non-brushed group. It must be concluded, therefore, as Ludford had himself suggested, that the reduction in tumour formation which followed his application of sandpaper, in place of the present

method of brushing the skin, had led to destruction rather than to stimulation of the epithelial cells which had come under the preparatory influence of the carcinogen.

SUMMARY.

Two hundred young inbred male mice were painted with 0·3 per cent. 1 : 2 : 5 : 6-dibenzanthracene in acetone once weekly for 16 weeks, and were then divided into two equal groups. During the next 33 weeks, prolonged mechanical irritation to the painted areas in the form of a twice weekly application of a stiff brush significantly increased both the formation of papillomas and carcinomas in that group. At this stage a further period of painting with 0·3 per cent. dibenzanthracene twice weekly for 16 weeks did not quite bring up the tumour yield in the controls to the original level obtained in the brushed group before the second period of painting was begun. The significance of these results is briefly discussed.

We wish to record our thanks to Prof. J. R. Learmonth, University of Edinburgh, for his interest throughout this investigation.

REFERENCES.

Deelman, H. T.—(1923–24) *Z. Krebsforsch.*, **21**, 220.
Des Ligneris, M. J. A.—(1940) *Amer. J. Cancer*, **40**, 1.
Eggers, H. E.—(1932) *Arch. Path.*, **13**, 112.
Lauridsen, J., and Eggers, H. E.—(1943) *Cancer Res.*, **3**, 43.
Ludford, R. J.—(1929) *Brit. J. exp. Path.*, **10**, 193.
Orr, J. W.—(1938) *J. Path. Bact.*, **46**, 495.
Seelig, M. G., and Cooper, Z. K.—(1933) *Amer. J. Cancer*, **17**, 589.